IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	)	
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Avraham COHEN et al.	)	Group Art Unit: 1625
	)	
Application No.: 10/507,485	)	Examiner: Margaret M. Seaman
	)	
Filed: September 13, 2004	)	Confirmation No.: 8571
	)	
For: ENANTIOMER (-) OF TENATOPROZOLE AND THE THERAPEUTIC USES THEREOF	)	

DECLARATION OF GEORGE SACHS PURSUANT 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, George Sachs, declare as follows:

1. I reside at 17986 Boris Drive Encino, CA 91316.

2. I am a citizen of the United States of America.

3. My educational background is as follows:

University of Edinburgh	1957	B.Sc., Biochemistry
University of Edinburgh	1960	M.B., Ch.B., Medicine
University of Edinburgh	1980	D.Sc. Biochemistry
University of Gothenburg	1987	M.D., Medicine

After / 3

4. I am a Professor of Medicine and Physiology, Wilshire Chair in Medicine, and Director of Membrane Biology Lab at the University of California, Los Angeles and a Staff Physician at the Los Angeles VA Greater LA Healthcare System. I have been employed by the University of California, Los Angeles since 1982.

5. I have served on the Center for Ulcer Research and Education Executive Committee and Advisory Board since 1982 and I have been the director and co-director of the Center for Ulcer Research and Education.

6. I am a named author on more than three hundred (300) journal publications (peer-reviewed), more than sixty (60) published reviews, six (6) text books, seven (7) letters, and three (3) editorials.

7. My research interests are in the field of gastroenterology and the microbiology of *H. pylori*. Specifically, my research interests include membrane transport processes, pump mechanisms, epithelial cell function, and bacterial bioenergetics.

8. A copy of my complete Curriculum Vitae is attached as Exhibit A.

9. I am not an inventor of U.S. Patent Application Serial No. 10/507,485. However, I am familiar with the patent application, as well as the experimental data that has been generated with regard to the metabolism of tenatoprazole (namely, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridyl)methyl]sulfinyl]imidazo[4,5-b]pyridine), including the racemate, the R-enantiomer (the (+) enantiomer), and the S-enantiomer (the (-) enantiomer).

Background

10. Proton pump inhibitors (PPIs) are usually used for treating various acid-related diseases of the upper gastro-intestinal tract, especially gastro-oesophageal reflux disease (GERD). After accumulation at the level of the gastric parietal cell and after chemical rearrangement, proton pump inhibitors bind covalently to the enzyme responsible for the transport of protons into the gastric juice, and hence, irreversibly inhibit gastric acid secretion.

Tenatoprazole

11. Tenatoprazole is the result of research aimed at prolonging the plasma half-life (*i.e.*, residence time in the blood) of PPIs, through manipulations of the benzimidazole structure. Tenatoprazole is an imidazopyridine derivative, with an increased plasma half-life in humans.

12. The clinical development of the tenatoprazole racemic mixture has demonstrated the clinical significance of the long half-life. The inhibition of acid secretion caused by tenatoprazole was sustained throughout the night and thus, has resulted in earlier efficacy and relief in patients with GERD. Studies have provided evidence of non-proportionality between the augmentation of pharmacokinetic parameters and the increase in dose of racemic tenatoprazole. A high inter-subject variability of the pharmacokinetics and of the pharmacodynamic response (level of acid inhibition) has been seen. For example, one subject presented a six-fold increase in the exposure (*i.e.*, the area under the plasma concentrations/time curve (AUC)).

13. This observation triggered the implementation of a specific study to identify the human cytochromes involved in the metabolism of tenatoprazole racemate, the (+) enantiomer, and the (-) enantiomer.

In vitro Identification of Cytochromes involved in (+) and (-) Enantiomer Metabolism

14. An *in vitro* study of cytochromes involved in the metabolism of the (+) enantiomer and the (-) enantiomer of tenatoprazole (TU-199) was performed. I have reviewed the results of this study in detail. Data from the study is attached as Exhibit B.

15. The results of the *in vitro* metabolism study identified the human cytochromes involved in the metabolism of the (+) enantiomer and the (-) enantiomer of tenatoprazole, using cDNA-expressed human CYPs. It was determined that CYP 2C19 is involved in 80 % of the metabolism of (+) enantiomer of tenatoprazole. For the (-) enantiomer, it was determined that CYP 2C19 was involved in only 53.4 % of the metabolism, and importantly, it was identified that CYP 3A4 was involved in 27.3 % of the metabolism and CYP 2C9 was involved in 19.3 % of the metabolism of this enantiomer.

16. Accordingly, CYP 2C19 is the dominant pathway for the metabolism of the (+) enantiomer of tenatoprazole. In contrast, pathways other than CYP 2C19 exist for metabolism of the (-) enantiomer (*i.e.*, CYP 3A4 and CYP 2C9).

17. Subjects with a genetic deficiency of the CYP 2C19 pathway (so called "poor metabolizers") account for the observed high inter-subject variability of the pharmacokinetics and of the pharmacodynamic response (level of acid inhibition) when dosing with the tenatoprazole racemate and the (+) enantiomer. These "poor metabolizers" have increased half-life and exposure (*i.e.*, the area under the plasma concentrations/time curve (AUC)), raising potential safety concerns.

18. In contrast, the (-) enantiomer has "escape" metabolic pathways in subjects with a genetic deficiency of the CYP 2C19 pathway (*i.e.*, CYP 3A4 and CYP 2C9). Therefore, even these "poor metabolizer" subjects can metabolize the (-) enantiomer. As such, the (-) enantiomer

exhibits a predictable half-life and exposure even in these "poor metabolizers" and therefore, can be dosed more safely and predictably in all subjects.

19. The incidence of so-called "poor metabolizers" is about 3 % in Caucasians and over 20 % in Asians and 6% in Hispanics.

Study of single and repeated oral administration of tenatoprazole racemate and enantiomers

20. A double blind, placebo controlled study on the tolerability of single and repeated dosing of tenatoprazole racemate (TU-199), as well as the (+) and (-) enantiomers, in healthy males was performed. I have reviewed the results of this study in detail. Data from the study is attached as Exhibit B.

21. In this study, the tenatoprazole racemate (TU-199), as well as the (+) and (-) enantiomers of tenatoprazole, were administered to sets of eight subjects per dose. The drug was administered once per day on day 1 of the study (the single administration phase) and then administration was resumed at once per day on days 14-20 (the repeated administration phase). C_{max} (maximum concentrations), T_{max} (maxiumum time), AUC (area under the curve) and $t_{1/2}$ (half-life) were determined for the racemate, as well as the (+) and (-) enantiomer. Values for "poor metabolizers" were not included in this data.

22. In the data in attached Exhibit B, it is seen that the pharmacokinetics of the racemate are not linear, and it is seen that the pharmacokinetics of the (+) enantiomer also are not linear. However, it is surprisingly seen that the pharmacokinetics of the (-) enantiomer are linear. Since the pharmacokinetics of the (-) enantiomer are linear, the non-linearity of the tenatoprazole racemate is due specifically to the (+) enantiomer.

23. The non-linear profile of the (+) enantiomer and the racemate result in unpredictability and reduction in safety in dosing in all patients since there is no proportionality between the increase in dose and increase in plasma concentrations. In contrast, the (-) enantiomer exhibits a linear profile providing predictability in dosing in all patients and increased safety since there is proportionality between the increase in dose and increase in plasma concentrations.

24. The metabolic by-products of the (+) enantiomer exhibit an inhibitory effect on the metabolism of the (+) enantiomer. This data further shows that the (+) enantiomer, and the racemate, are unpredictable in patient dosing.

Study of pharmacokinetics of tenatoprazole racemate and enantiomers in extensive and poor metabolizers

25. An open-label parallel-group study on the pharmacokinetics of the tenatoprazole racemate (TU-199), the (+) enantiomer, and the (-) enantiomer after single oral dose administration in extensive and poor CYP2C19 metabolizers was performed. I have reviewed the results of this study in detail. Data from the study is attached as Exhibit B. The "extensive metabolizers" do not have the genetic deficiency of the CYP 2C19 pathway and are considered "normal" subjects.

26. In this study, the tenatoprazole racemate (TU-199), as well as the (+) and (-) enantiomers of tenatoprazole, were administered to eight healthy volunteers (four poor metabolizers and four extensive metabolizers). The volunteers were given a single 20 mg oral dose under fasting conditions. The study period was 192 hours after administration for the poor metabolizers and 72 hours after administration for the extensive metabolizers. The C_{max}, T_{max}, AUC and $t_{1/2}$ of TU-199 (+) enantiomer, and (-) enantiomer were determined.

27. When comparing the C_{max}, T_{max}, AUC and $t_{1/2}$ for the racemate and the (+) enantiomer for the poor metabolizers and the extensive metabolizers, drastic differences are observed. However,

for the poor metabolizers and the extensive metabolizers, unexpectedly, there is no significant pharmacokinetic variation in the C_{max}, T_{max}, AUC and $t_{1/2}$ for the (-) enantiomer.

28. The mean exposure (AUC) to tenatoprazole racemate (TU-199) was found to be over four-fold higher in poor metabolizers in comparison to extensive metabolizers. The elimination half-life ($t_{1/2}$) of the tenatoprazole racemate (TU-199) was about 30 hours in poor metabolizers, compared to 6 hours in extensive metabolizers.

29. Unexpectedly, this difference is due to the (+) enantiomer. The mean exposure (AUC) to the (+) enantiomer was found to be over 21-fold higher in the same poor metabolizers in comparison to the same extensive metabolizers (*i.e.*, 173,996 ng/h/mL^{-1} vs. 8,085 ng/h/mL^{-1}). In addition, the half-life ($t_{1/2}$) was increased by eight-fold (36.7 vs. 4.5 hours).

30. In contrast, the (-) enantiomer showed only a slightly increased half-life in the same poor metabolizers in comparison to the same extensive metabolizers (*i.e.*, 9.7 vs. 5.7 hours).

Conclusions

31. Unexpectedly, the pharmacokinetics of (-) enantiomer are linear in both subjects without a genetic deficiency of the CYP 2C19 pathway ("normal subjects") and in subjects with a genetic deficiency of the CYP 2C19 pathway ("poor metabolizers"). The linearity allows for predictability in dosing in all patients, thus providing increased safety in dosing in all patients.

32. Also unexpectedly, the pharmacokinetics of (+) enantiomer are not linear in either subjects without a genetic deficiency of the CYP 2C19 pathway ("normal subjects") or in subjects with a genetic deficiency of the CYP 2C19 pathway ("poor metabolizers"). The metabolic by-products of the (+) enantiomer exhibit an inhibitory effect on the metabolism of the (+) enantiomer. Possible safety concerns arise from the poor predictability in dosing in all

patients, the increased half life and exposure in patients, and the increased potential for drug interactions.

33. In summary, the (-) enantiomer has a predictable pharmacodynamic response, due to its linear pharmacokinetic response to dosing. The racemate and the (+) enantiomer are not predictable in this way. The (-) enantiomer showed a lower variability in metabolism in both subjects without a genetic deficiency of the CYP 2C19 pathway ("normal subjects") or in subjects with a genetic deficiency of the CYP 2C19 pathway ("poor metabolizers"). Therefore, the (-) enantiomer could be administered to any subject, without considering the subject's CYP 2C19 polymorphism status.

34. Moreover, it is my opinion that it is unexpected that the (-) enantiomer of tenatoprazole exhibits such a different pharmacokinetic profile, in comparison to the tenatoprazole racemate and the (+) enantiomer.

I further declare that all statements made herein of my own knowledge are true and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 10/26/05

George Sachs

CURRICULUM VITAE

PERSONAL HISTORY:

NAME: George Sachs

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BIRTHDATE: August 26, 1935

PLACE OF BIRTH: Vienna, Austria

CITIZENSHIP: U.S.A.

EDUCATION:

University of Edinburgh	1957	B.Sc., first class honors	Biochemistry
University of Edinburgh	1960	M.B., Ch.B., with honors	Medicine
University of Edinburgh	1980	D.Sc.	Biochemistry
University of Gothenburg	1987	M.D., (Hon Causa)	Medicine

LICENSURE: California, Physician and Surgeon #A 54208

PROFESSIONAL EXPERIENCE:

PRESENT POSITIONS:

University of California, Los Angeles	1982-present	Professor, Medicine & Physiology
University of California, Los Angeles	1982-present	Wilshire Chair in Medicine
University of California, Los Angeles	1987-present	Director, Membrane Biology Laboratory
VAGLAHS-West Los Angeles	1999-present	Staff Physician

PREVIOUS POSITIONS:

Albert Einstein College	1961-1962	Instructor
Columbia University	1962-1963	Research Associate

University of Alabama in Birmingham	1963-1965	Assistant Professor, Medicine & Physiology
University of Alabama in Birmingham	1965-1970	Associate Professor, Medicine & Physiology
University of Alabama in Birmingham	1970-1982	Prof. Medicine & Physiology
University of Alabama in Birmingham	1974-1982	Director, Membrane Biology
University of Alabama in Birmingham	1979-1982	Professor, Medicine & Physiology & Biophysics
University of California, Los Angeles	1982-1987	Director, Center for Ulcer Research & Education
VAGLAHS-West Los Angeles	1984-1999	Senior Medical Investigator
University of California, Los Angeles	1987-2003	Co-Director, Center for Ulcer Research & Education

PROFESSIONAL ACTIVITIES:

COMMITTEE SERVICES:

National:
National Committee Biophysical Society, 1976-1978
NSF Panel for Metabolic Biology, 1977-1979
American Physiological Society GI Steering Committee, 1977-1981
NIH Study Section, Physiology, 1979-1983
National Board Medical Examiners, 1979-1983
VA Merit Review Board, 1984-1987
National Committee Biophysical Society, 1987-1989
NIAMS, Board of Scientific Counselors, NIH, 1993
NIH Study Section NIDDK Digestive Disease Centers, 2000-Present
NIH Advisory Board, 2002
NIH Study Section NIDDK Digestive Disease Centers, 2002-Present
NIH Special Study Section, 2003-Present

Local:
Center for Ulcer Research and Education Executive Committee, 1982-Present
Center for Ulcer Research and Education Advisory Board, 1982-Present
Veterans Administration Review Committee, 1983-1986
IBD/Harbor UCLA Advisory Board, 1988-1992
Chair, Academic Personnel Committee, UCLA, 1990-1991
GI/UCLA Search Committee, 1992-1993
UCLA Specialty Training and Academic Research Committee (STAR), 1993-1995
UCLA, Academic Personnel ad hoc Review Committee, 1994
VAMC, Wadsworth, ACOS for Research Search Committee, 1994
VAMC, West Los Angeles R&D, GI and Hepatic Disorder Review Group, 1996
VAMC, West Los Angeles Merit Review Committee, 1996
VAMC, West Los Angeles Internal Merit Review Committee, 1996
VAGLAHS-West Los Angeles, Chair, Research Sub-Committee, 2000-Present

PROFESSIONAL ASSOCIATIONS:

American Gastroenterological Association
American Physiological Society

American Society for Biochemistry and Molecular Biology
American Society of Biological Chemistry
American Society for Microbiology
American Society of Renal Biochemistry and Metabolism
Biochemical Society
British Gastroenterological Association (Honorary)
Society of General Physiology

EDITORIAL SERVICES:

American Journal of Physiology, Editorial Board, 1968-1977
American Journal of Physiology, Associate Editor, 1977-1985
Hypertension, Editorial Board, 1982-1985
Physiological Reviews, Editorial Board, 1983-1989
Annual Review of Physiology, Associate Editor, 1985-1990
American Journal of Physiology, Editorial Board, 1985-Present
American Heart Association, Review Board, 1988-1991
Alimentary Pharmacology & Therapeutics, Editorial Board, 1988-2004
Digestive Diseases and Sciences, Editorial Board, 1990-Present
Frontiers of Bioscience, Editorial Board, 2001
World Journal of Gastroenterology, Editorial Board, 2004-Present
Reviewer, Biochemistry, 2004-Present

HONORS AND SPECIAL AWARDS RECEIVED:

Humboldt Award for U.S. Senior Scientists, 1973-1974
Hoffman LaRoche Award, 1982
Senior Medical Investigatorship, Veterans Administration, 1984-1999
Beaumont Prize in Gastroenterology, American Gastroenterological Association, 1985
Middleton Award, Veterans Administration, 1992
"Ismar Boas Vorlesung" Medal, German Gastroenterological Association, 1992
Fifth "Morton I. Grossman Distinguished Lectureship", 1993
Honorary Membership, British Society of Gastroenterology, 1993
Distinguished Lecturer, Department of Pharmacology, University Texas Medical School at Houston, 1995
Outstanding Scientific and Technical Award, Federal Executive Board of Los Angeles, 1996
Honorary Degree, Doctor of Medicine, Medical Faculty of Gothenburg University, Sweden, 1996
Horace W. Davenport Distinguished Lecturer of the APS Gastrointestinal Section, 1998
Janssen Award for Special Achievement in Gastroenterology, 1998
Outstanding Scientific and Technical Award, Federal Executive Board of Los Angeles, 1998
Outstanding Supporter, Upward Bound Internship Program Harvey Mudd College, 2000
Gairdner Foundation Awardee, 2004
Dr. Norman Frankel Scholar to the University of Chicago, 2005

BIBLIOGRAPHY

Journal Publications (Peer-Reviewed):

1. G. Sachs and O. Braun-Falco. The occurrence and nature of arylsulfatases in parakeratoses. *J.Invest Dermatol.* 34:439-444, 1960.
2. G. Sachs, C. Deduve, B. S. Dvorkin, and A. White. Effect of adrenal cortical steroid injection on lysosmal enzymic activities of rat thymus. *Exp.Cell Res.* 28:597-600, 1962.
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5. B. I. Hirschowitz and G. Sachs. Vagal gastric secretory stimulation by 2-deoxy-D-glucose. *Am.J.Physiol* 209 (3):452-460, 1965.
6. G. Sachs and B. I. Hirschowitz. Effect of diisopropyl fluorophosphate on gastric secretion and gastric ATPase. *Proc.Soc.Exp.Biol.Med.* 120 (3):702-704, 1965.
7. G. Sachs, R. Shoemaker, and B. I. Hirschowitz. Action of 2-deoxy-D-glucose on frog gastric mucosa. *Am.J.Physiol* 209 (3):461-466, 1965.
8. G. Sachs, W. E. Mitch, and B. I. Hirschowitz. Frog gastric mucosal ATPase. *Proc.Soc.Exp.Biol.Med.* 119 (4):1023-1027, 1965.
9. B. I. Hirschowitz and G. Sachs. Reversal of insulin inhibition of gastric secretion by intravenous injection of potassium. *Am.J.Dig.Dis.* 11 (3):217-230, 1966.
10. G. C. Luketic, G. Sachs, J. Myren, T. Tsuji, and B. I. Hirschowitz. Effects of colchicine on intestinal mucosal dehydrogenases. II. Biochemical observations. *Am.J.Dig.Dis.* 11 (5):404-409, 1966.
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12. G. Sachs, R. L. Shoemaker, and B. L. Hirschowitz. Effects of sodium removal on acid secretion by the frog gastric mucosa. *Proc.Soc.Exp.Biol.Med.* 123 (1):47-52, 1966.
13. R. L. Shoemaker, G. Sachs, and B. I. Hirschowitz. Secretion by guinea pig gastric mucosa in vitro. *Proc.Soc.Exp.Biol.Med.* 123 (3):824-827, 1966.
14. B. I. Hirschowitz and G. Sachs. Insulin inhibition of gastric secretion: reversal by ribidium. *Am.J.Physiol* 213 (6):1401-1405, 1967.
15. B. I. Hirschowitz and G. Sachs. Insulin effects on gastric secretion and blood electrolytes modified by injected potassium. *Am.J.Dig.Dis.* 12 (1):7-18, 1967.
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18. G. Sachs, R. Shoemaker, and B. I. Hirschowitz. The action of amytal on frog gastric mucosa. *Biochim.Biophys.Acta* 143 (3):522-531, 1967.
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TENATOPRAZOLE

A double blind placebo controlled study on the tolerability of single and repeated oral administrations of tenatoprazole *(racemate)* in healthy male Caucasian subjects

PKC DATA - PHASE I

TENATOPRAZOLE

PHARMACOKINETIC PROTOCOL

AIM

To assess pharmacokinetic profile of single and repeated oral doses of TU-199 in healthy male caucasian volunteers

- Determination of CMAX; TMAX; AUC; T1/2; accumulation ratio
- Linearity assessment

NUMBER OF SUBJECTS

8 subjects per dose including 2 placebo for plasma, and 8 subjects per dose including 2 placebo for urine.

DOSES : 10, 20, 40, 80 and 120 mg

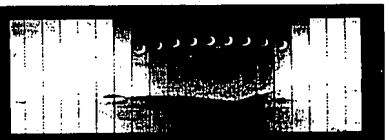
TREATMENT REGIMEN

Drug is given on the morning under fasting conditions :

on Day 1 (single administration phase)

from Day 14 to 20 (repeated administration phase)

TENATOPRAZOLE



TU-199 : Single - Repeated dose

TU-199 - Day 1: Single

	Cmax		Tmax		AUC t		AUC τ		AUC inf		t 1/2	
	ng.mL	sd	h	sd	ng.h.mL	sd	ng.h.mL	sd	ng.h.mL	sd	h	sd
10 mg	865,4	165,5	3,8	1,8	10440,0	7414,0	7787,0	1477,0	10688,0	7632,0	9,4	11,4
10 mg *	891,3	170,8	3,4	1,7	7429,2	858,4	7267,0	838,0	7586,8	845,4	4,8	1,2
20 mg	2411,7	615,0	4,3	1,4	23975,0	10054,0	21865,0	8131,0	24273,0	10318,0	6,2	1,1
40 mg	5284,7	2173,7	2,5	0,8	42563,0	15813,0	39833,0	13903,0	42881,0	15892,0	5,6	1,4
80 mg	8248,2	2135,5	3,2	1,0	97169,0	49577,0	82136,0	35302,0	97651,0	49621,0	7,3	2,3
120 mg	15645,4	4779,0	3,5	1,5	219670,0	131008,0	175072,0	64420,0	220350,0	130838,0	7,7	3,0

TU-199 - Day 20: Repeated

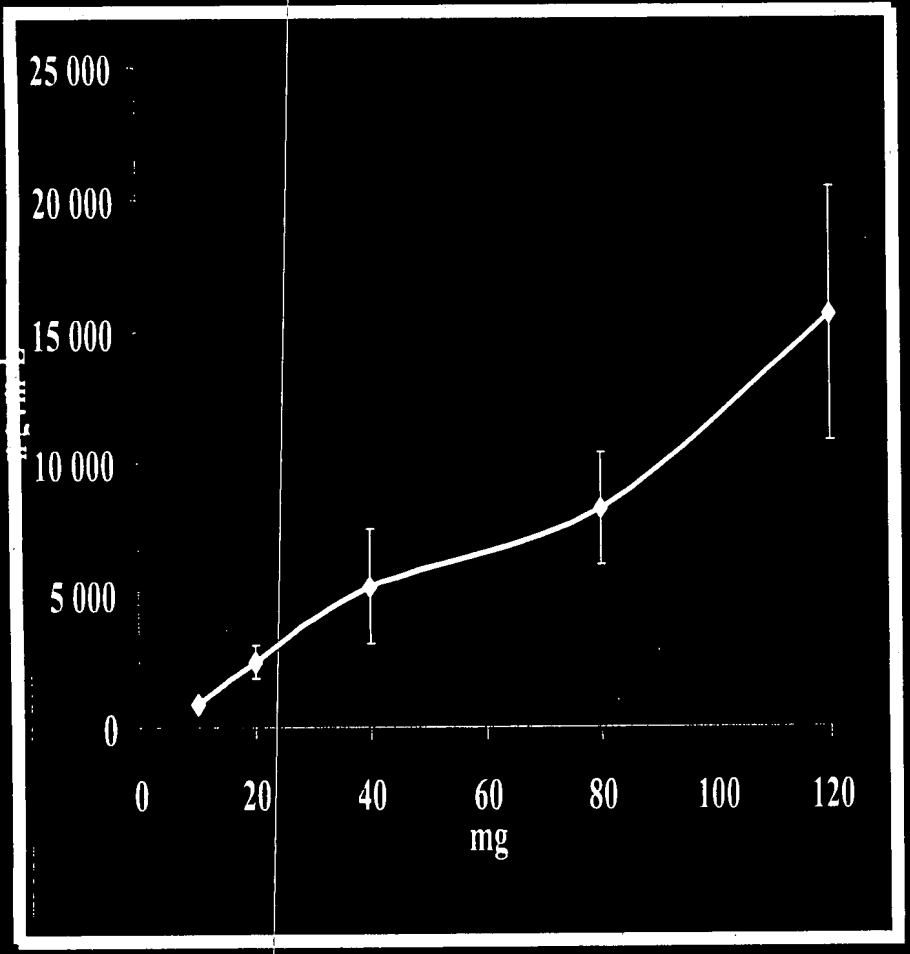
	Cmax		Tmax		AUC t		AUC τ		AUC inf		t 1/2	
	ng.mL	sd	h	sd	ng.h.mL	sd	ng.h.mL	sd	ng.h.mL	sd	h	sd
10 mg	1641,3	923,2	2,7	1,0	35349,0	55776,0	20213,0	20379,0	36577,0	58227,0	10,4	12,9
10 mg *	1284,2	330,6	2,6	1,1	12675,0	5746,0	12090,2	4926,0	12897,6	5715,1	5,2	0,6
20 mg	2989,9	789,5	2,2	0,4	35910,0	18793,0	31003,0	13056,0	36267,0	18745,0	7,8	2,0
40 mg	5494,6	1201,6	3,3	3,0	75240,0	27629,0	61489,0	16941,0	75448,0	27626,0	8,7	2,6
80 mg	11819,3	2831,9	2,3	0,8	218372,0	108840,0	157327,0	60252,0	218867,0	108578,0	9,2	2,3
120 mg	30196,5	14036,9	2,2	1,2	517588,0	232580,0	375958,0	132851,0	517915,0	232600,0	8,8	1,9

*** values without the poor metabolizer**

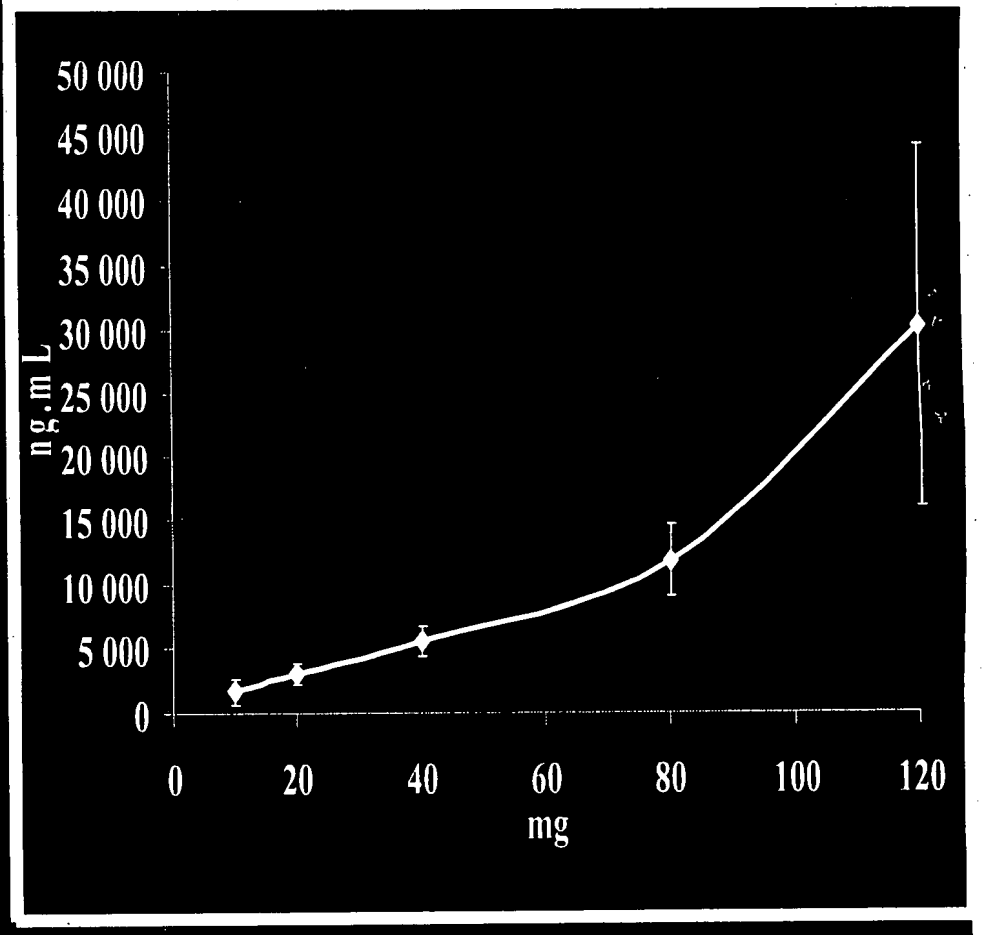
TENATOPRAZOLE

C_{MAX} (maximal concentrations) (TU-199 – *racemate*)

SINGLE ADMINISTRATION



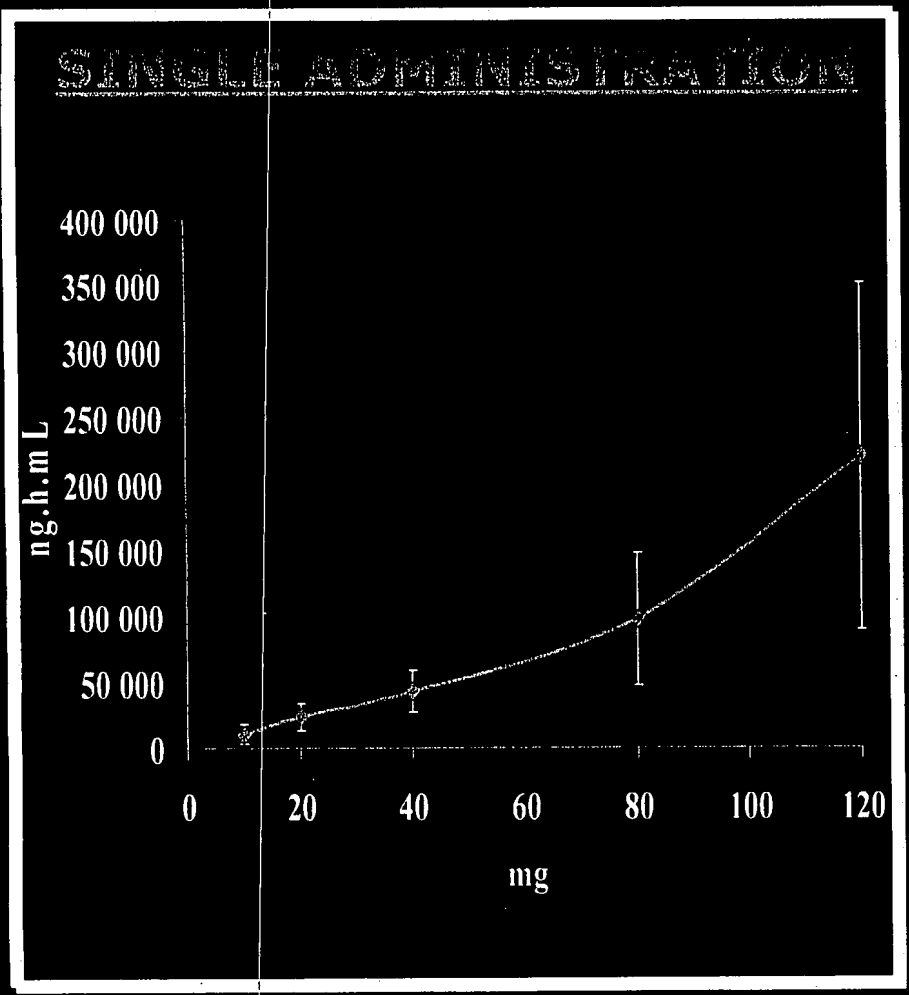
REPEATED ADMINISTRATION

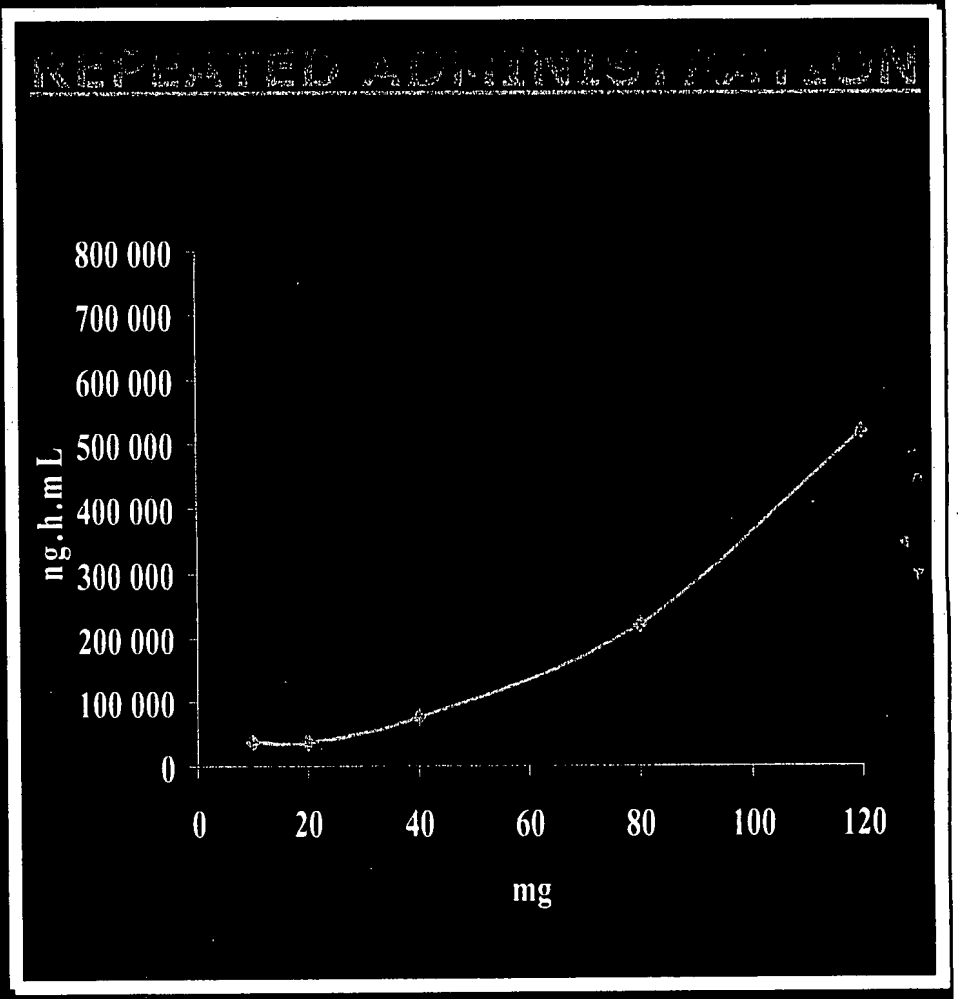


Non-linearity from 10 to 120 mg

TENATOPRAZOLE

AUCinf *(exposure)* (TU-199 – *racemate*)

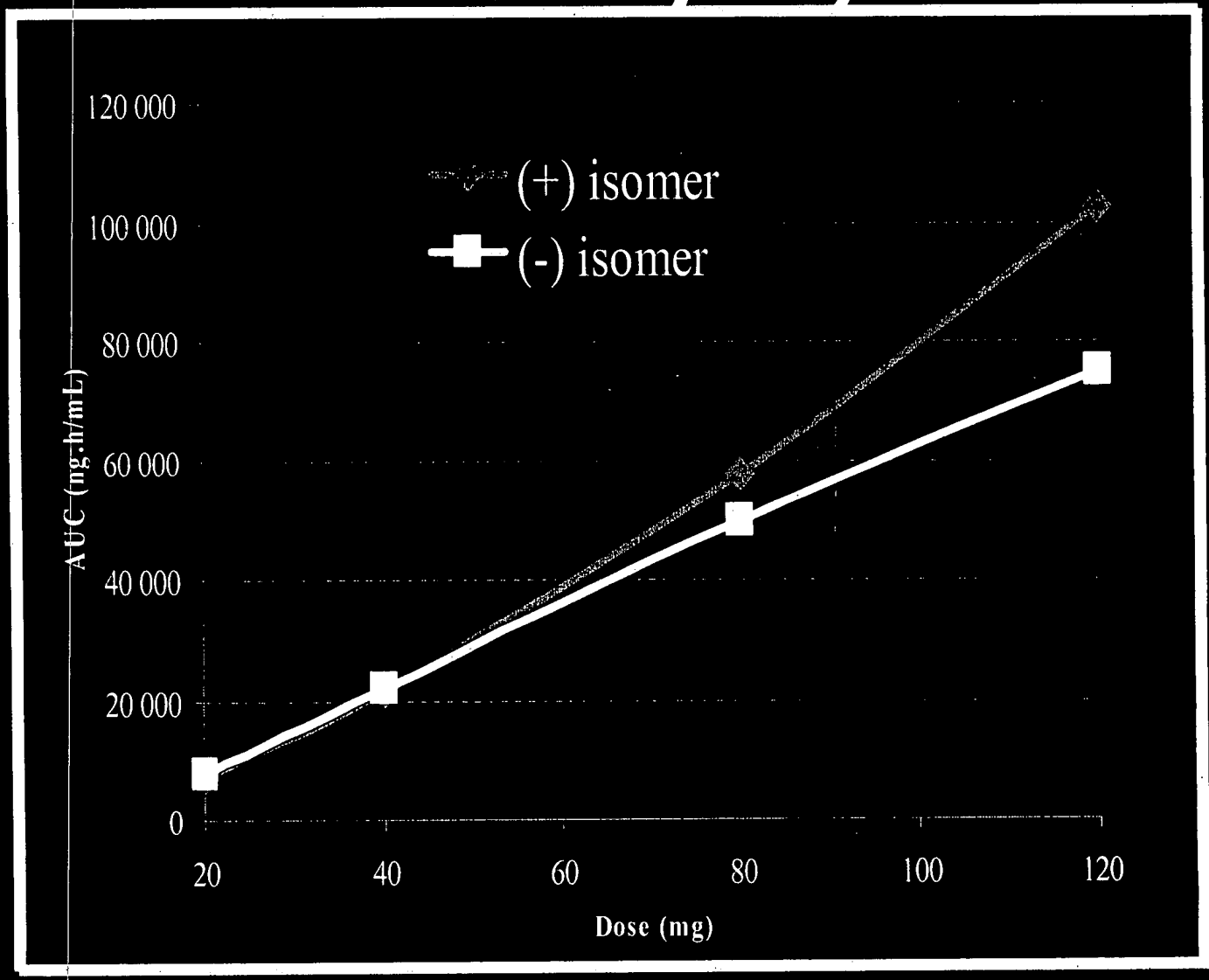




Non-linearity from 10 to 120 mg

TENATOPRAZOLE

AUCinf *(exposure)* (+) and (-) enantiomers (Chiral analysis)



CONCLUSION

In humans, the pharmacokinetics of TU-199 *(racemate)* are not linear (no proportionality between the increase in dose and increase in plasma concentrations, thus raising a safety concern

This is due – unexpectedly – to the (+) enantiomer, whereas pharmacokinetics of (-) enantiomer are linear.

TENATOPRAZOLE

An open-label, parallel-group study to evaluate the pharmacokinetics of TU-199 *(racemate)* after single oral dose administration in extensive and poor CYP2C19 metabolizers

PKC DATA - PHASE I

TENATOPRAZOLE

PHARMACOKINETIC PROTOCOL

AIM

- To evaluate the pharmacokinetics of TU-199 (racemic and enantiomers) and its metabolites after single oral 20 mg dose administration in fast and slow CYP2C19 metabolizers;
- To evaluate possible difference in pharmacodynamics (gastrinemia) between the 2 groups.

NUMBER OF SUBJECTS

8 healthy volunteers (4 poor metabolizers and 4 extensive metabolizers).

DOSE : 20 mg.

TREATMENT REGIMEN

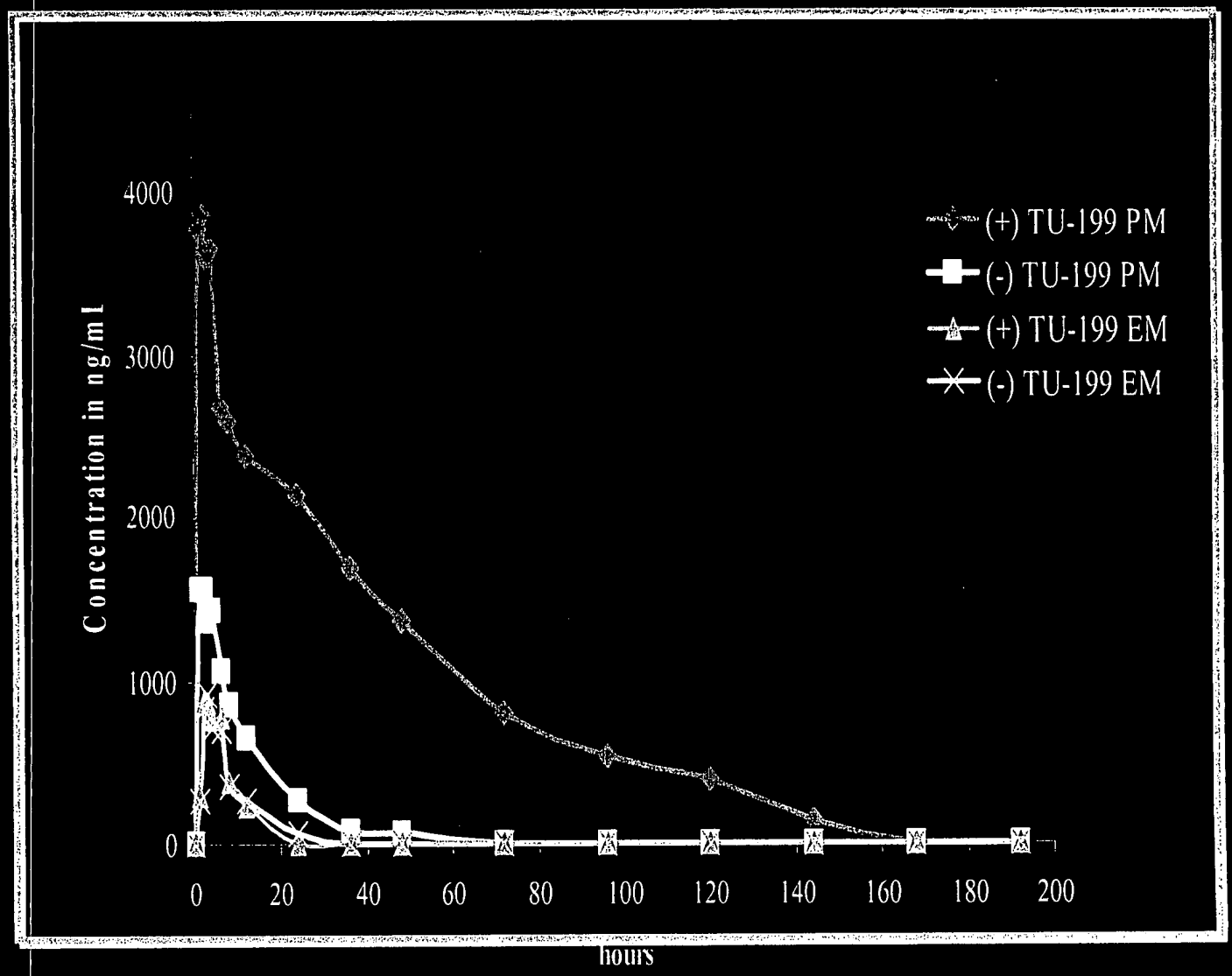
A single oral dose of 20 mg is given on the morning under fasting conditions.

STUDY PERIOD

192 hrs after administration for poor metabolizers (PM) and 72 hrs for extensive metabolizers (EM).

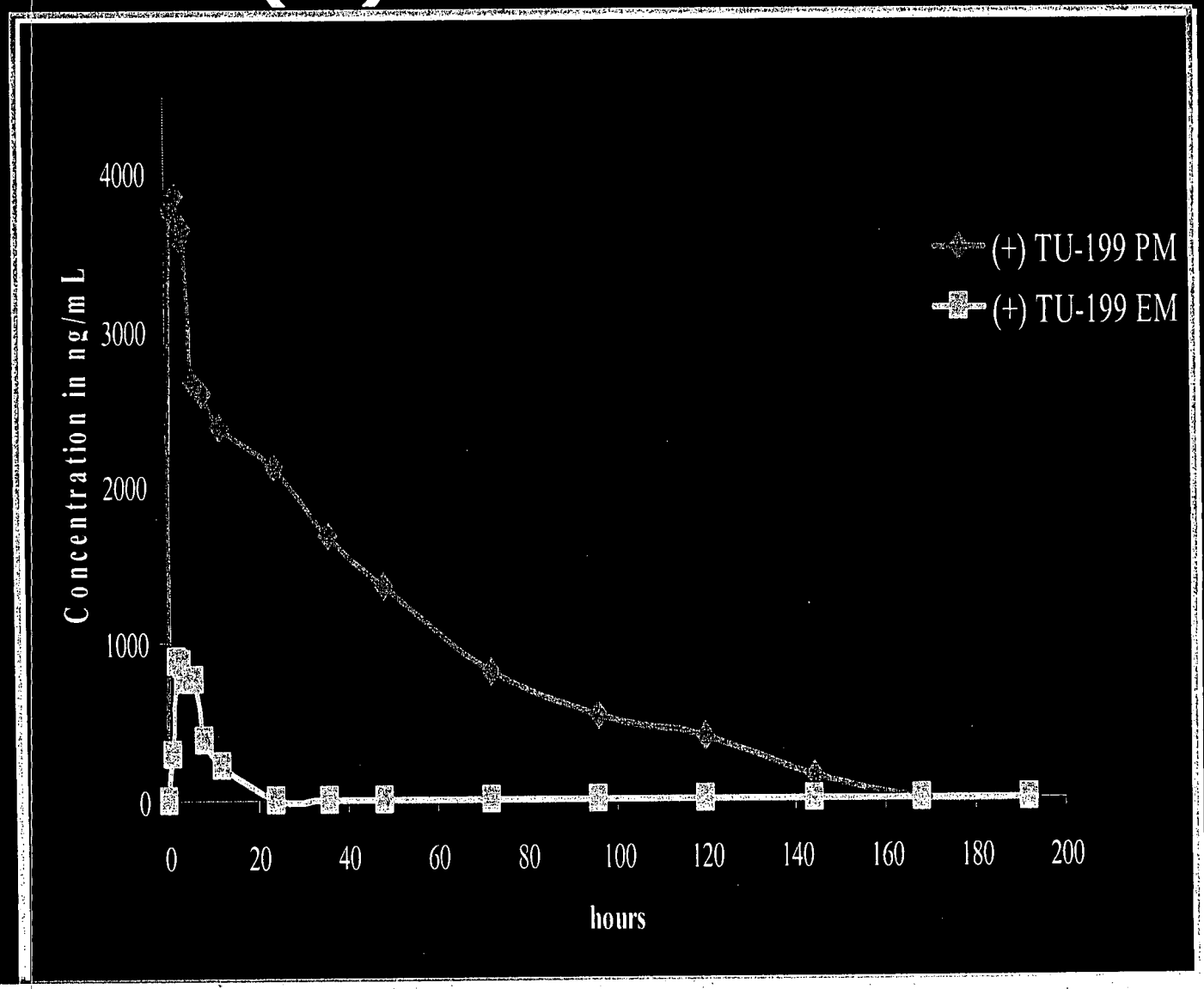
TENATOPRAZOLE

C_{MAX} (maximal concentrations) (+) and (-) enantiomers



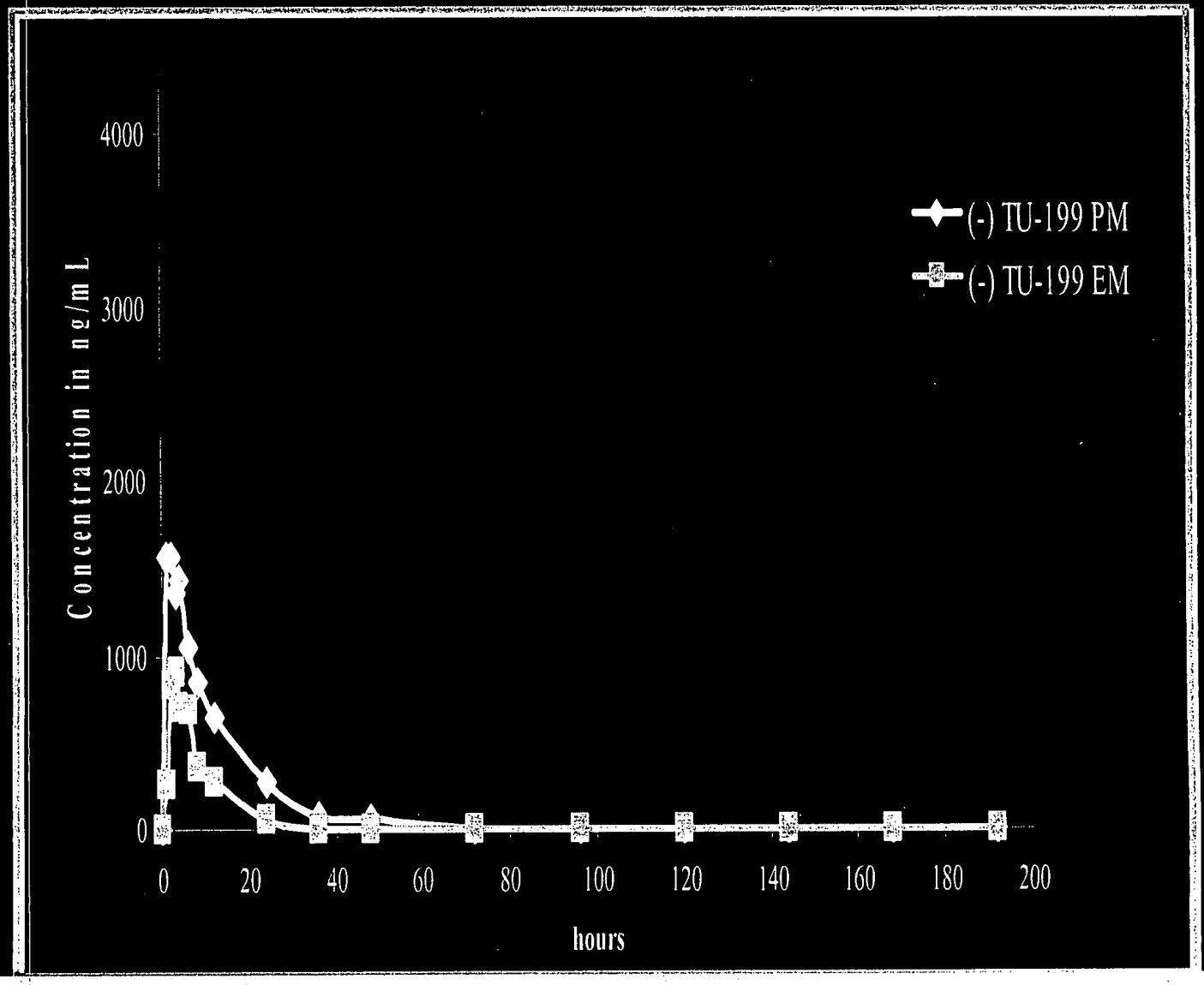
TENATOPRAZOLE

C_{MAX} (maximal concentrations) (+) enantiomer



TENATOPRAZOLE

C_{MAX} (maximal concentrations) (-) enantiomer



TENATOPRAZOLE

RESULTS

Parameters	TU-199		(+) TU-199		(-) TU-199	
	PM	EM	PM	EM	PM	EM
Cmax (ng/mL)	3069.60 ± 302.29	2104.11 ± 410.18	4306.01± 449.66	1201.14 ± 220.95	1752.57 ± 225.81	1130.90 ± 243.27
Tmax (h)	1.8 ± 1.0	3.3 ± 1.9	1.3 ± 0.5	3.3 ± 1.9	1.3 ± 0.5	3.3 ± 1.9
Median (Min/Max)	1.5 (1.0 – 3.0)	2.5 (2.0 – 6.0)	1.0 (1.0 – 2.0)	2.5 (2.0 – 6.0)	1.0 (1.0 – 2.0)	2.5 (2.0 – 6.0)
AUCt (ng.h/mL)	87477 ± 9047	20841 ± 2322	162314 ± 17851	7303 ± 1555	20767 ± 4488	7883 ± 2658
AUCinf (ng.h/mL)	88508 ± 9212	21015 ± 2371	173996 ± 13352	8085 ± 1480	22309 ± 3902	8633 ± 2756
t1/2 (h)	30.07 ± 2.06	5.76 ± 0.36	36.74 ± 4.55	4.46 ± 1.04	9.74 ± 0.89	5.74 ± 1.82

Dramatic changes between PMs and EMs

Spread data due to (+) enantiomer

No significant pharmacokinetic variation ☞ easy to manage

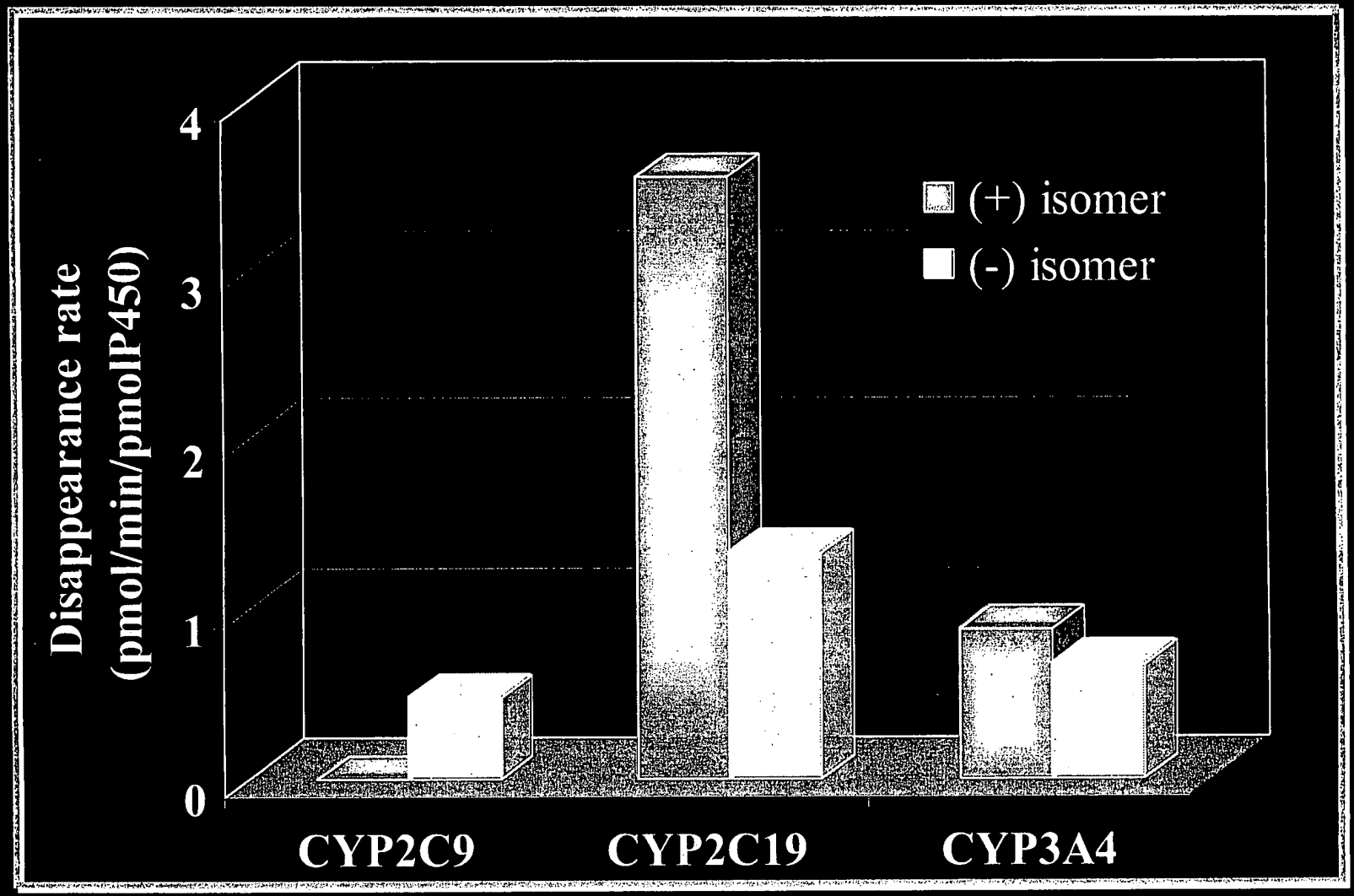
CONCLUSION

In poor metabolizers, both elimination half-life and area under the concentrations/time curve (AUC – exposure) for TU-199 *(racemate)* are about 5-fold increased, compared to extensive metabolizers.

This is due – unexpectedly – to the (+) enantiomer.

TENATOPRAZOLE

IN-VITRO IDENTIFICATION OF CYTOCHROMES INVOLVED IN (+) an (-) ENANTIOMERS METABOLISM



TENATOPRAZOLE

(+) TU-199		(-) TU-199
• Intrinsic clearance (2C19)	> (about 5 times)	• Intrinsic clearance
• Role of CYP 2C19	>	• Role of CYP 2C19
• Half life in PM	>>>	• Half life in PM

Difference between EM and PM
+ + +

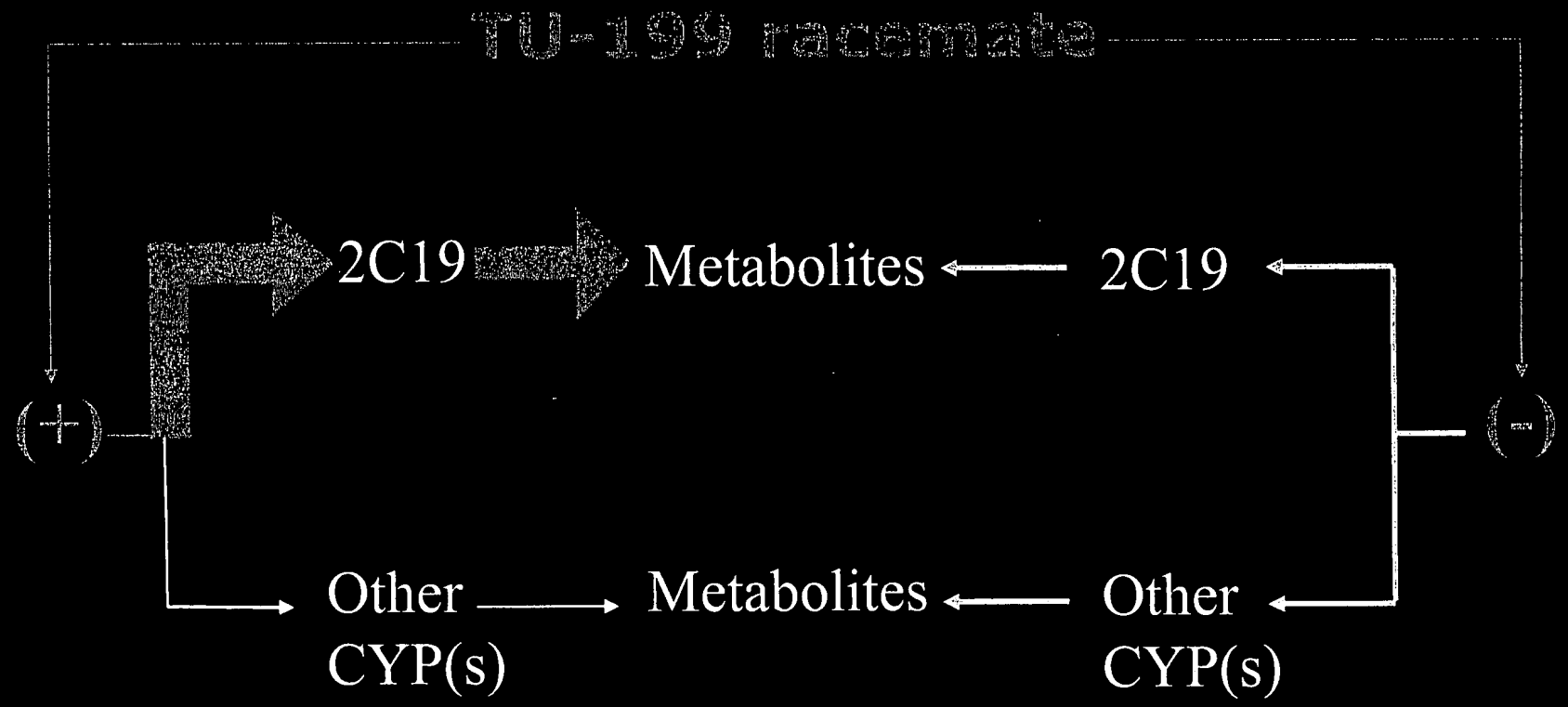
Difference between EM and PM very low

Better prediction

Higher safety

TENATOPRAZOLE

TU-199 METABOLIC SCHEME IN EXTENSIVE METABOLIZERS

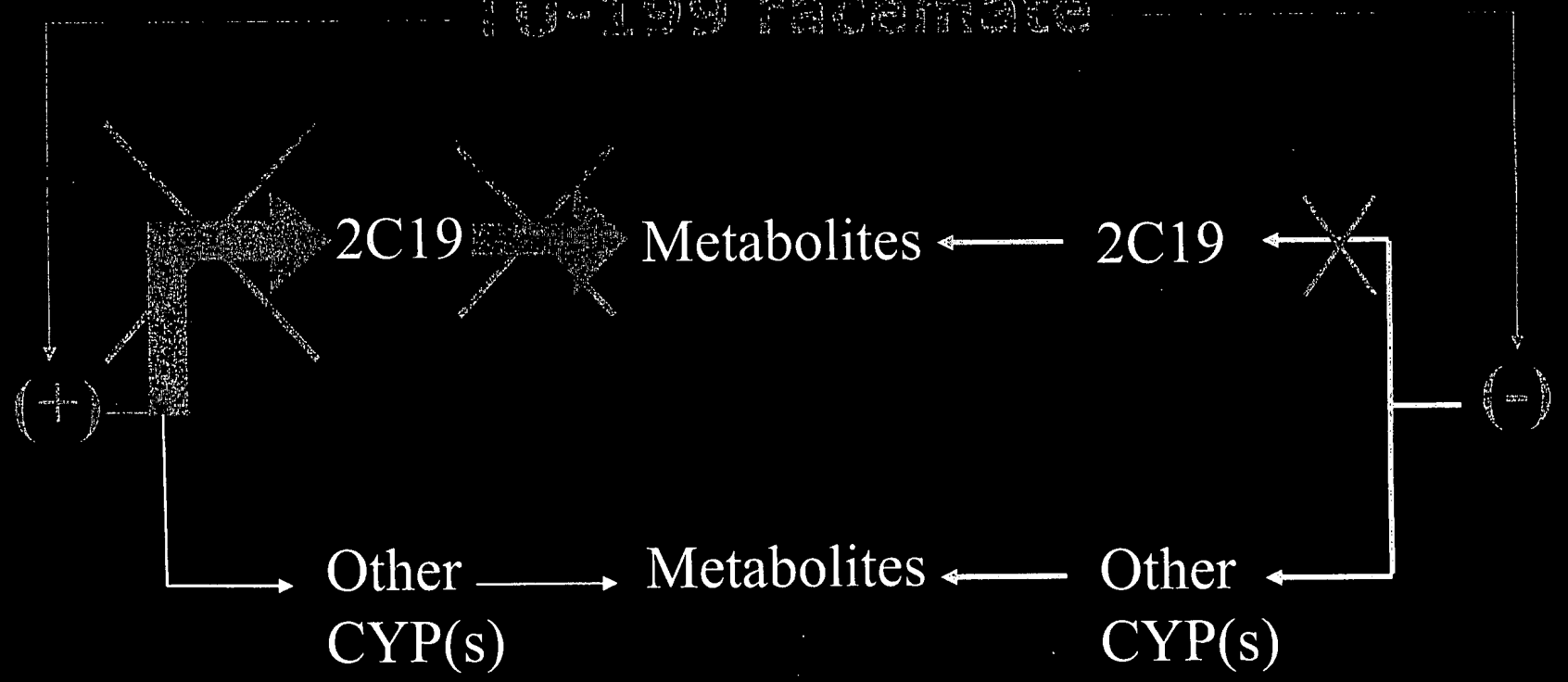


Main pathway for (+) enantiomer is CYP 2C19

Other CYP pathways exist for (-) enantiomer: CYP 3A4, CYP 2C9

TENATOPRAZOLE

TU-199 METABOLIC SCHEME IN POOR METABOLIZERS



Increased long-half life and exposure in vivo, due to genetic deficiency of main pathway

« Escape » metabolic pathways exist, even when CYP 2C19 is genetically deficient

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Characterization of the inhibitory activity of tenatoprazole on the gastric H^+,K^+-ATPase in vitro and in vivo

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Omeprazole

Abbreviations:
DTT, 1,4-dithio-DL-threitol
F-MI, fluorescein-5-maleimide
GERD, gastro-esophageal reflux disease
GSH, glutathione
H2RA, H_2-receptor antagonist
NEM, *N*-ethyl maleimide
PPI, proton pump inhibitor
TM, transmembrane segment
TPZ, tenatoprazole

ABSTRACT

Tenatoprazole is a prodrug of the proton pump inhibitor (PPI) class, which is converted to the active sulfenamide or sulfenic acid by acid in the secretory canaliculus of the stimulated parietal cell of the stomach. This active species binds to luminally accessible cysteines of the gastric H^+,K^+-ATPase resulting in disulfide formation and acid secretion inhibition. Tenatoprazole binds at the catalytic subunit of the gastric acid pump with a stoichiometry of 2.6 nmol mg^{-1} of the enzyme in vitro. In vivo, maximum binding of tenatoprazole was 2.9 nmol mg^{-1} of the enzyme at 2 h after IV administration. The binding sites of tenatoprazole were in the TM5/6 region at Cys813 and Cys822 as shown by tryptic and thermolysin digestion of the ATPase labeled by tenatoprazole. Decay of tenatoprazole binding on the gastric H^+,K^+-ATPase consisted of two components. One was relatively fast, with a half-life 3.9 h due to reversal of binding at cysteine 813, and the other was a plateau phase corresponding to ATPase turnover reflecting binding at cysteine 822 that also results in sustained inhibition in the presence of reducing agents in vitro. The stability of inhibition and the long plasma half-life of tenatoprazole should result in prolonged inhibition of acid secretion as compared to omeprazole. Further, the bioavailability of tenatoprazole was two-fold greater in the (S)-tenatoprazole sodium salt hydrate form as compared to the free form in dogs which is due to differences in the crystal structure and hydrophobic nature of the two forms.

1. Introduction

Proton pump inhibitors (PPIs) are now widely used for treatment of both erosive and non-erosive gastro-esophageal reflux disease (GERD and NERD) [1]. Current PPIs used clinically are substituted pyridylmethylsulfinyl benzimidazole prodrugs. They accumulate in the acidic secretory canaliculus of the parietal cell (luminal surface of the gastric ATPase) and then undergo an acid-catalyzed chemical rearrangement, resulting in an active thiophilic species, a sulfenic acid or sulfenamide, that binds to various cysteines accessible from the luminal surface of the gastric H^+,K^+-ATPase, forming

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disulfides [2,3]. Recent chemical evidence suggests that the PPI protonated on the pyridine nitrogen binds to the pump and then undergoes activation to the sulfenic acid due to a second protonation on the benzimidazole nitrogen [3]. The efficacy of these drugs depends on their covalent binding to the pump so that their effect far outlasts their plasma residence time above threshold, which is about 10–120 min. However, at the time of administration, the parietal cell must be secreting acid, since this is required for their activation from prodrug to the active species. Given the relatively short residence time and the fact that not all pumps are activated by breakfast or other meals, the effect of the drugs is cumulative, reaching steady state on once a day dosing after about 3 days. In principle, benefit would be achieved with a PPI with a longer residence time, as appears to be the case with esomeprazole, the S-enantiomer of omeprazole. Tenatoprazole (TPZ) has a residence time of about 9 h, suggesting it might show even superior efficacy, especially at night.

Tenatoprazole (TU-199), 5-methoxy-2-(4-methoxy-3,5-dimethyl-pyridin-2-ylmethanesulfinyl)-1H-imidazo[4,5-*b*]pyridine, is a novel proton pump inhibitor which is similar to the chemical structure of omeprazole, a widely used proton pump inhibitor. The difference between tenatoprazole and omeprazole is that tenatoprazole has an imidazo[4,5-*b*]pyridine moiety, where omeprazole has a benzimidazole moiety. This reduces the rate of metabolism, allowing a longer plasma residence time but also decreases the pK_a of the fused-imidazole N as compared to the current PPIs (Fig. 1).

Tenatoprazole belongs to the class of covalent proton pump inhibitors and inhibits gastric H^+,K^+-ATPase with potency similar to omeprazole. However, the anti-secretory and anti-ulcer effects of tenatoprazole were reported to be two to four times more potent than those of omeprazole with long-lasting effects on gastric acid secretion [4,5]. In human studies, tenatoprazole was more potent than esomeprazole. Particularly, the pH > 4 holding time was higher during the night for tenatoprazole than for esomeprazole. Hence, the duration of nocturnal acid breakthrough was significantly less for tenatoprazole than for esomeprazole [6]. Tenatoprazole exerted a more potent acid inhibition than esomeprazole during first 48 h in healthy volunteers. These differences resulted from better night-time acid control with tenatoprazole 40 mg than esomeprazole 40 mg. The duration of nocturnal acid breakthroughs was significantly reduced for both night-time periods [7]. Furthermore, tenatoprazole provided a prolonged duration of acid suppression and a shorter nocturnal acid breakthrough in healthy volunteers, even after stopping the drug [8].

Previously, we had investigated the reversal of in vivo inhibited gastric H^+,K^+-ATPase by treating the enzyme isolated from PPI-treated rats with disulfide reducing agents and measuring recovery of ATPase activity as a function of time of incubation in vitro and the different binding property between omeprazole and pantoprazole [9,10]. Two factors are considered relevant in terms of their efficacy, their biological target, the gastric acid pump that is the final step in acid secretion, and their covalent binding to cysteines of the acid pump that provides duration of action much longer than that predicted from their plasma half-life. The sites of binding of different PPIs varied between binding at Cys813 (omeprazole) and Cys813 and Cys822 (pantoprazole). Binding of pantoprazole at Cys822 was stable with a half-life similar to the enzyme turnover, while binding at Cys813 was sensitive to glutathione, providing a shorter half-life of binding at this site. Inhibition was retained due to the second binding site at cysteine 822, in the membrane domain of the ATPase [9,10]. Binding of PPI at Cys822 was related to a slower activation rate of pantoprazole as compared to omeprazole [3]. Tenatoprazole activation was slower than pantoprazole at pH 1.3, which suggests that tenatoprazole may also access Cys822.

In this study, in vitro and in vivo inhibitory activity of tenatoprazole with identification of binding sites was investigated. Also, we studied the residence time of binding in vivo, and reversal of tenatoprazole binding by a reducing agent ex vivo. We also investigated the basis for the differential solubility of the sodium salt of S-tenatoprazole as compared to the racemate or the free base.

2. Materials and methods

2.1. Materials

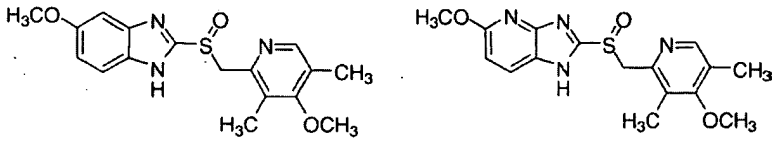
Hog stomachs were obtained from local slaughter house, Farmer-Johns. Hog gastric H^+,K^+-ATPase was prepared from the fundic mucosa of the stomach. Male rats (Sprague–Dawley, 200–250 g) were used. ^{14}C-tenatoprazole, ^{14}C-(*R*)-tenatoprazole, and ^{14}C-(*S*)-tenatoprazole were gifts of Negma-Lerads, Les Hameaux Cedex, France. All reagents were analytical grade or higher.

2.2. Animals

The animal study was approved by the Animal Care and Use Committee of VA Greater Los Angeles Healthcare System and fulfilled National Institutes of Health guidelines for use of animal subjects. Male rats (Sprague–Dawley, 200–250 g) were used.

2.3. Hog gastric H^+,K^+-ATPase enzyme preparation

The gastric H^+,K^+-ATPase was prepared from hog gastric mucosa by previously published methods, which involve



Omeprazole Tenatoprazole

Fig. 1 – Chemical structure of omeprazole and tenatoprazole.

differential and density gradient centrifugation [11]. The gastric fundic mucosa was scraped from the stomach and then homogenized in a solution of 0.25 M sucrose, 5 mM Pipes/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA. The homogenate was centrifuged at 11,000 rpm in a Sorvall GSA rotor for 45 min. The supernatant was centrifuged at 34,000 rpm in a Beckman type 35 rotor for 1 h. The microsomal pellet was resuspended in a solution of 0.25 M sucrose, 5 mM Pipes/Tris, pH 6.8, 1 mM EDTA, and 1 mM EGTA. The microsomal suspension was further purified using a Z-60 zonal rotor [12]. The vesicles obtained have been shown to be over 90% cytoplasmic side out and ion tight. The potassium impermeability of the vesicles was determined by the difference in K^+ stimulation of ATPase activity in the presence of KCl alone and in the presence of KCl and the potassium ionophore, nigericin. The specific activity in the presence of nigericin was 120 μmol ATP hydrolyzed mg^{-1} protein h^{-1}, and in the absence of nigericin, 10 μmol mg^{-1} h^{-1}. Thus, greater than 90% of the K^+-stimulated ATPase activity was dependent on the addition of nigericin indicating K^+ impermeability of 90% of the hog gastric vesicles.

2.4. Characterization of in vitro inhibition of tenatoprazole

2.4.1. Acridine orange uptake in hog gastric H^+,K^+-ATPase enzyme

Acridine orange uptake was measured by a previously described method [13]. The enzyme suspension (20 μg ml^{-1}) was incubated at 37 °C in a buffer composed of 5 mM Pipes/Tris (pH 6.95), 2 mM $MgCl_2$, 150 mM KCl, 3 μg ml^{-1} of valinomycin, 1 μM acridine orange, in the presence of 0.1 mM glutathione, in a SPEX spectrofluorometer for 5 min. Inhibitor (20 μM), omeprazole or tenatoprazole, was added and fluorescence was measured by excitation at 490 nm and emission at 530 nm. After 60 s, ATP (2 mM) was added to initiate acridine orange uptake as a measure of intra-vesicular acidification.

2.4.2. In vitro inhibition of tenatoprazole on the gastric H^+,K^+-ATPase

The inhibition was carried out as follows. The enzyme (4 μg ml^{-1}) was incubated at 37 °C for 1 h in a buffer composed of 5 mM Pipes/Tris (pH 6.6), 2 mM $MgCl_2$, ±150 mM KCl, 1 μg ml^{-1} of valinomycin, 2 mM ATP, in the presence of 0.1 mM glutathione and inhibitor (0, 0.2, 0.5, 1, 2, 5, and 10 μM). The ATPase activity of the enzyme was measured in the presence of nigericin as follows. 0.5 ml of a buffer composed of 5 mM Pipes/Tris (pH 6.6), 2 mM $MgCl_2$, 4 μg ml^{-1} of nigericin, 4 mM [γ-^{32}P]ATP, and 0.1 mM glutathione was added to the control or inhibited enzyme suspension (0.5 ml) to initiate generation of inorganic phosphate [^{32}Pi]. The final composition of the enzyme reaction suspension was: enzyme (2 μg ml^{-1}), 5 mM Pipes/Tris (pH 6.6), 2 mM $MgCl_2$, ±75 mM KCl, 0.5 μg ml^{-1} of valinomycin, 2 μg ml^{-1} of nigericin, 2 mM [γ-^{32}P]ATP with <1 mM ATP, and 0.1 mM glutathione. The enzyme suspension was incubated for 30 min and the reaction was stopped by adding ice-cold 1 ml ammonium molybdate solution (four parts of 4.5% ammonium molybdate and one part of 70% perchloric acid). Ice-cold butyl acetate (2 ml) was added and vortexed to extract the inorganic phosphate. The butyl acetate layer was separated from aqueous layer by centrifugation and an aliquot of butyl acetate (1 ml) was taken out for counting. Basal Mg-ATPase activity was measured in the same mixture in the absence of KCl and background ATP hydrolysis was measured in the absence of added enzyme. Gastric H^+,K^+-ATPase activity was calculated by subtracting Mg-ATPase activity and background ATP hydrolysis from the activity in the presence of K^+, and Mg-ATPase activity was obtained by subtracting background ATP hydrolysis from the activity obtained in the absence of K^+. The IC_{50} of tenatoprazole, omeprazole, or esomeprazole was determined.

2.4.3. Determination of in vitro binding stoichiometry of tenatoprazole

The stoichiometry of tenatoprazole binding was determined on an enzyme preparation that consisted of 85% ATPase as follows. The gastric H^+,K^+-ATPase (20 μg ml^{-1}) was incubated at 37 °C for 0.5 h in a buffer composed of 5 mM Pipes/Tris (pH 6.6 or 7.0), 2 mM $MgCl_2$, ±150 mM KCl, 3 μg ml^{-1} of valinomycin, 2 mM ATP, in the presence of 0.1 mM glutathione and inhibitor (20 μM). Glutathione does not penetrate the intact vesicles and prevents non-acid activated binding to the outside surface of the acid-transporting vesicles.

The reaction mixture was centrifuged at 100,000 × *g* for 1 h and the membrane pellet was resuspended in a buffer composed of 50 mM Tris/HCl, pH 7.0, at 1 mg ml^{-1} protein. An aliquot was taken out to measure the enzyme activity. The other aliquot was taken out to measure binding of [^{14}C]-tenatoprazole to the enzyme. In a typical run, an aliquot containing 20 μg of the enzyme was precipitated by adding nine-fold excess of ice-cold methanol and incubating on ice for 30 min. The radioactivity of the precipitated protein was determined to measure the nanomole of tenatoprazole bound per milligram of the H^+,K^+-ATPase.

2.4.4. Identification of regions of tenatoprazole binding site by trypsin cleavage

The gastric H^+,K^+-ATPase (0.2 mg ml^{-1}) was incubated at 37 °C in a solution composed of 0.25 M sucrose, 150 mM KCl, 50 mM Tris/HCl, pH 7.0, 2 mM $MgCl_2$, 2 μg ml^{-1} valinomycin, 1 mM EGTA/Tris, 2 mM ATP, 1 mM glutathione, and 10 μM C^{14}-tenatoprazole for 30 min. The enzyme suspension was centrifuged at 110,000 × *g* for 1 h. The pellet was resuspended in 0.25 M sucrose, 50 mM Tris/HCl, pH 8.2, at a concentration of 1 mg ml^{-1} with the addition of 0.2 mg ml^{-1} trypsin. The digestion was stopped by addition of 10× trypsin inhibitor. These trypsin digested membranes were centrifuged at 110,000 × *g* for 1 h, and the pellet was washed with 50 mM Tris/HCl, pH 8.2, and resuspended in 50 mM Tris/HCl, pH 8.2, at a protein concentration of 1 mg ml^{-1}. An aliquot was treated with 1% SDS and 0.2 mM fluorescein maleimide (F-MI) to determine unreacted cysteines in the transmembrane segments. The digested membranes were size fractionated by SDS-PAGE using 12–20% tricine gradient gels. All fluorescent bands detected were separated and sequenced and radioactivity measured.

2.4.5. Identification of tenatoprazole-binding sites by thermolysin digestion

To further identify the site of labeling by tenatoprazole in the TM5/6 region, additional digestion was carried out as

described previously [10,13]. Here, [^{14}C] tenatoprazole-labeled gastric vesicles (0.8 mg) were extensively digested with trypsin (0.2 mg) in a buffer (1 ml) composed of 0.25 M sucrose, 50 mM Tris/HCl, pH 8.2, for 60 min. The reaction was stopped by adding 2 mg of soybean trypsin inhibitor. The membrane digest was spun in a Beckman L5 centrifuge using a Ti-65 rotor at 100,000 × *g* for 60 min. The pellet was labeled with fluorescein-5-maleimide (F-MI) by dissolving in a buffer (0.8 ml) composed of 20 mM Tris/HCl, pH 6.7, 0.2 mM fluorescein-5-maleimide, and 0.5% SDS. The F-MI was used here again to identify any unreacted cysteines. The membrane digest sample was combined with an electrophoresis sample buffer and applied to a 10–20% tricine big slab gel and run at 50 mA constant current as previously described [10,13]. The lowest region at 4.5–6.2 kDa molecular weight range contained a fluorescent band representing the TM1/2 segment and the [^{14}C] tenatoprazole-labeled TM5/6 segment. This band was sliced from the gel. The labeled peptide fragments were electroeluted from the gel slices using Bio-Rad Electroeluter Model 422 equipped with a Membrane Caps (molecular cut-off 3.5 kDa). Electroelution was carried out in a buffer composed of 30 mM Tris/HCl, pH 8.0, 0.03% SDS at 120 V constant for 6 h. The electroelution buffer was carefully removed and replaced with a new buffer composed of 10 mM Tris/HCl, pH 8.0, 0.01% SDS. Electrodialysis was carried out at 100 V constant for 1 h. The eluate was separated and diluted by adding three-fold excess volume of a buffer composed of 10 mM Tris/HCl, 3 mM $CaCl_2$, pH 8.0. The eluate was concentrated by filtration through an Amicon Ultra Centrifugal Filter Device-4500 MWCO (Millipore) down to 0.2 ml. The eluate was divided into two portions. One portion served as a control, and the other portion was digested further with 30 μg of thermolysin (Sigma, Protease Type X) at 37 °C for 24 h to further cleave TM5/6. After digestion, the samples were combined with 10 μl of 2 M sucrose and 0.25% bromphenol and placed on top of a 1.5-mm gradient slab gel 16% (17:1 acrylamide/methylene bisacrylamide) gel. The gel was run in the cold room (4 °C) for 18 h at 45 mA constant current along with a lane for CNBr-cleaved fragments of horse myoglobin (Sigma, 17–2.5 kDa) as molecular weight standards. The peptides were transferred electrophoretically to PVDF membranes (Millipore) for 18–24 h in the cold room (4 °C) in a tank transfer apparatus at 120 mA constant current, in a transfer buffer of 150 mM glycine, 20 mM Tris, and 20% methanol. A sandwich of three sheets of Whatman 3-mm filter paper was placed on either side of the gel, which had a prewetted PVDF membrane on the anode side. After transfer, the blots were rinsed twice in distilled water and stained with 0.1% Coomassie Blue in 10% glacial acetic acid and 45% methanol. In every case, a duplicate lane was run to provide material for sequencing as well as for either counting or autoradiography. Reducing agents were absent in all experiments since these remove the bound PPI. Standard curves of $\ln(M_r)$ as a function of relative mobility were used to estimate the M_r of the peptide products of digestion. The accuracy of the M_r determination appeared to be within 10% based on predicted tryptic cleavage sites within the primary sequence of catalytic subunit of the enzyme.

2.5. *Characterization of in vivo inhibition of tenatoprazole*

2.5.1. *Preparation of crude gastric membrane containing rat gastric H^+,K^+-ATPase labeled by ^{14}C-S(−)-tenatoprazole, ^{14}C-R(+)-tenatoprazole, and ^{14}C-(R,S)-tenatoprazole*

Rats were fasted for 24 h with free access to water. Rats were maximally stimulated by subcutaneous histamine (40 mg kg^{-1}) and carbachol (20 μg kg^{-1}) injection and radioactive ^{14}C-(R)-tenatoprazole, ^{14}C-(S)-tenatoprazole, or ^{14}C-(R,S)-tenatoprazole were administered by intravenous injection at 0.1 mCi kg^{-1} in each animal with a dosage of 20 μmol kg^{-1} ($N = 4$–6 per each group) through the tail vein. The rats were sacrificed at timed intervals, 1, 2, 4, 6, 12, and 24 h. The stomachs were opened and washed with a phosphate buffered saline (pH 7.4) buffer. The corpus was separated, blotted with paper, and kept in a 15 ml borosilicate bottle on ice. From each rat, the corpus mucosa was scraped and the tissue was then resuspended in 3.5 ml of a homogenization buffer composed of 0.25 M sucrose, 2 mM EDTA, 2 mM EGTA, and 10 mM Pipes/Tris, pH 7.4. The mucosal suspension was homogenized with 15 strokes of Teflon pestle at 2000 rpm in a Potter–Elvehjem homogenizer. All operations were done at 2–4 °C. The homogenate was centrifuged at 1600 × *g* for 30 min at 4 °C, and the pellet was discarded. The supernatant was centrifuged at 100,000 × *g* at 4 °C for 60 min to give the crude membrane pellet. This membrane pellet was resuspended in 2 ml of a buffer composed of 0.25 M sucrose, 10 mM Pipes/Tris, pH 7.4. About 12% of the membrane protein was the gastric H^+,K^+-ATPase.

2.5.2. *Quantification of the H^+,K^+-ATPase in the crude gastric membranes*

The amount of gastric H^+,K^+-ATPase in each crude membrane fraction was quantified by the method described previously [10]. Briefly, an aliquot of the membrane fraction (1 μg) was run on a mini slab SDS-gel (8%) together with given amounts of hog gastric H^+,K^+-ATPase membranes (0.05–0.3 μg) which are about 85% pure. The quantity of H^+,K^+-ATPase was determined by Western blotting of the membranes with the monoclonal antibody Ab12.18 and by comparing the staining intensity in the membrane fraction with the known amounts of hog gastric H^+,K^+-ATPase. Staining intensity was analyzed by using the software, Image J program (Version 1.29x, Wayne Rasband, National Institutes of Health, USA).

2.5.3. *Reversal of H^+,K^+-ATPase activity*

An aliquot of ^{14}C-tenatoprazole labeled enzyme was resuspended in a buffer composed of 50 mM Tris/HCl, pH 7.4, in the presence or absence of 10 mM glutathione (GSH) or dithiothreitol (DTT), at a H^+,K^+-ATPase protein concentration of 0.02 mg ml^{-1}. For incubation of the microsomal membranes with glutathione as a reducing reagent, the vesicles were made leaky by five cycles of freezing and thawing. These enzyme aliquots were incubated in the presence of 10 mM reducing reagent at 37 °C. At timed intervals up to 1 h, the potassium-stimulated gastric H^+,K^+-ATPase activity was measured.

The ATPase assay was performed at 37 °C by measurement of P_i released from ATP using butyl acetate extraction as above.

Basal K^+-stimulated activity was determined in the absence of reducing reagents at zero time of incubation.

A 50 μl aliquot was taken from each incubation with 10 mM reducing reagent and added to the K^+-activity assay buffer (950 μl) composed of 20 mM KCl, 50 mM Tris/HCl, pH 7.0, 2 mM $MgCl_2$, 2 μg ml^{-1} nigericin, 1 mM EGTA/Tris, 400 μM ouabain, 1 μM oligomycin, 10 nM bafilomycin, and 1 mM ATP. These inhibitors are added to reduce non-specific ATPase activity and nigericin added to ensure K^+ permeability of the resting membrane fraction. Basal Mg-ATPase activity was measured in the same mixture in the absence of KCl and background ATP hydrolysis measured in the absence of added enzyme. All assays were performed at 37 °C for 30 min and stopped by adding ice-cold 1 ml stop solution (one part of 60% perchloric acid and four parts of 4.5% ammonium molybdate) on ice. Butyl acetate (2 ml) was added and the reaction mixture extracted. An aliquot of butyl acetate (1 ml) was taken out and used to measure free inorganic phosphate. All measurements were in triplicate. In the experiments with glutathione, the resting membranes were subjected to five cycles of freeze thawing to enable access of glutathione to the exoplasmic surface of the enzyme.

Gastric H^+,K^+-ATPase activity was calculated as above by subtracting Mg-ATPase activity and background ATP hydrolysis from the activity in the presence of K^+, and Mg-ATPase activity was obtained by subtracting background ATP hydrolysis from the activity obtained in the absence of K^+. Reversal of inhibition was expressed as percent control activity obtained after exposure to either 10 mM dithiothreitol or 10 mM glutathione.

2.5.4. *Effect of reducing agents on binding*

An aliquot of enzyme was resuspended in a buffer composed of 50 mM Tris/HCl, pH 7.4, in the presence or absence of 10 mM glutathione or dithiothreitol, at a H^+,K^+-ATPase protein concentration of 0.1 mg ml^{-1}. These enzyme aliquots were incubated at 37 °C. At timed intervals, an aliquot containing 15 μg of the H^+,K^+-ATPase was taken out and precipitated by adding nine-fold excess of ice-cold methanol and kept on ice for 20 min, then, centrifuged at maximum speed of an Eppendorf centrifuge for 2 min. The precipitated pellet was resuspended in 0.3 ml of ice-cold methanol, and centrifuged again to remove any residual non-bound PPI. The precipitated materials were dissolved in 0.3 ml of 50 mM Tris/HCl, 0.2% SDS, pH 7.0, and 1 mM N-ethyl maleimide (NEM), and counted for their ^{14}C isotope content.

2.5.5. *Identification of tenatoprazole-binding regions of rat gastric H^+,K^+-ATPase labeled in vivo*

Crude membranes containing 100 μg of the H^+,K^+-ATPase were diluted to 0.5 ml by adding 0.25 M sucrose, 0.2 M Tris/HCl, pH 8.2. Trypsin 80 μg dissolved in 80 μl of 0.1 M Tris/HCl, pH 8.2, was added and the mixture was incubated for 30 min at 37 °C. The samples were kept on ice and soybean trypsin inhibitor 800 μg dissolved in 100 μl of 50 mM Tris/HCl (pH 8.2) was added. The membranes were centrifuged at 100,000 × *g* for 1 h (at 4 °C). The pellet was briefly rinsed with 1 ml of 0.25 M sucrose, 0.1 M Tris/HCl, pH 7.4, then, dissolved in 400 μl of 0.01 mM NEM, 0.1 M Tris/HCl, pH 7.4, with 0.2% SDS. To this solution, 100 μl of sample buffer composed of 40% sucrose, 0.01% phenol blue, and 0.1 M Tris/HCl, pH 7.4, was added. One hundred microliters of sample having 25 μg of the H^+,K^+-ATPase was applied on a 10–20% tricine big slab gel and run at 50 mA constant. Hog gastric vesicles were treated by the same method as described above and run on one lane next to the crude membrane digest for comparing transmembrane segments. The gel was transblotted on to a PVDF membrane and a film was developed to obtain the autoradiogram from the PVDF blot. This method has been previously described in detail and has allowed identification of the different sites of labeling by proton pump inhibitors [10].

2.5.6. *Pharmacokinetics of (S)-tenatoprazole sodium salt, (S)-tenatoprazole free form, and (R,S)-tenatoprazole free form*

(R,S)-Tenatoprazole free form (50 mg kg^{-1}) or (S)-tenatoprazole sodium salt hydrate (50 mg kg^{-1}) was orally administered to dogs and the plasma level of tenatoprazole was measured as a function of time course. Similarly, (S)-tenatoprazole free form (100 mg kg^{-1}) or (S)-tenatoprazole sodium salt hydrate (100 mg kg^{-1}) was orally administered to dogs and the plasma level of tenatoprazole was measured as a function of time.

2.6. *Crystal structure of (R,S)-tenatoprazole free form and sodium salt hydrate, (S)-tenatoprazole free form and sodium salt hydrate form and their solubility*

2.6.1. *Crystal structure of (R,S)-tenatoprazole free form, (R,S)-tenatoprazole sodium salt hydrate, (S)-tenatoprazole free form, and (S)-tenatoprazole sodium salt form*

An X-ray powder diffraction spectrum was obtained using PANalytical X'pert pro diffractometer (PANalytical Inc.) and a single crystal was analyzed using Bruker Smart 1000 CCD diffractometer (Bruker-Axs Inc.).

Crystals of the compounds were prepared as follows. (R,S)-Tenatoprazole sodium salt dihydrate was completely dissolved in minimum volume of methanol in a vial loosely closed, and placed in a hood at room temperature until crystal growth. (S)-Tenatoprazole sodium salt hydrate was dissolved in ethyl acetate and ethanol with one drop of water in a vial without a cap. The vial was placed in a jar containing hexane, then, the jar was tightly closed and kept at room temperature until crystals growth. (R,S)-Tenatoprazole free form or (S)-tenatoprazole free form was crystallized from ethyl acetate.

2.6.2. *Solubility measurement of tenatoprazole free form and sodium salt form*

(R,S)-Tenatoprazole free form, (R,S)-tenatoprazole sodium salt hydrate, (S)-tenatoprazole free form, and (S)-tenatoprazole sodium salt form were dissolved in water with excess amounts of materials and filtered. The volumes were measured and dried in vacuo for 24 h. Residual materials were weighed. Solubility was determined as weight per volume. Alternatively, the solution filtered was diluted and its UV absorbance at 310 nm and the concentration were determined.

2.7. *Statistical analysis*

The results were expressed as mean values ± S.D.

3. Results

Tenatoprazole is 5-methoxy-2-(4-methoxy-3,5-dimethyl-pyridin-2-ylmethanesulfinyl)-1H-imidazo[4,5-*b*]pyridine and the main structural difference between tenatoprazole and omeprazole is that an imidazo[4,5-*b*]pyridine ring replaces the benzimidazole ring of omeprazole. This results in different chemical, physical, and biological properties of tenatoprazole. These differences result in different pharmacological activity of tenatoprazole as compared to omeprazole.

3.1. *In vitro inhibition by tenatoprazole*

3.1.1. *Inhibitory activity of tenatoprazole on the hog gastric H^+,K^+-ATPase*

When the gastric H^+,K^+-ATPase of the membrane vesicles pumps acid into the sealed vesicle interior, acridine orange, a weak base, is accumulated and the fluorescence is quenched due to stacking of the dye allowing measurement of acidification of the gastric vesicles as described previously [13]. When PPI becomes activated by acid, the active form of PPI inhibits enzyme activity, resulting in elevation of the intravesicular pH and restoration of the fluorescence of acridine orange. Omeprazole provided a faster restoration of acridine orange fluorescence than tenatoprazole (Fig. 2, Panel A). This shows that omeprazole inhibits proton transport faster than tenatoprazole. Fig. 2(Panel B) shows that the rate of inhibition of ATPase activity by omeprazole is faster than with tenatoprazole at given concentration below 2 μM. The IC_{50} measured at pH 6.6 was 0.4 μM for omeprazole and 3.2 μM for tenatoprazole. The previously determined IC_{50} of omeprazole was 0.47 μM [14].

3.1.2. *Stoichiometry of tenatoprazole of the gastric H^+,K^+-ATPase labeled in vitro*

Using hog gastric H^+,K^+-ATPase, inhibition by tenatoprazole was measured in the presence of glutathione to find the stoichiometry of tenatoprazole bound to the H^+,K^+-ATPase. In this experiment, the reaction medium pH was 7.0 and the incubation time was 30 min with 20 μM of inhibitor. Glutathione prevented non-selective binding of auto-activated tenatoprazole and reduced non-selective binding to the outside face of the enzyme.

Tenatoprazole labeled only the gastric H^+,K^+-ATPase alpha-subunit as has been found for other PPIs [10,13,15,16]. Table 1 summarizes the binding stoichiometry of tenatoprazole comparing binding and %inhibition. Approximately 2.6 nmol mg^{-1} of tenatoprazole was bound to the H^+,K^+-ATPase, the same was found for rabeprazole [17], pantoprazole, and omeprazole [10]. The two enantiomers (R)- or (S)-tenatoprazole gave the same stoichiometry of binding with 88% inhibition. There was virtually equal binding of the (R)-, (S)-, and (R,S)-forms of the compound.

3.1.3. *Identification of regions of binding by trypsin cleavage*

Tenatoprazole-labeled peptide fragments generated by trypsin digestion were separated by SDS-PAGE (Fig. 3). Tenatoprazole labeled only the peptide containing fifth and sixth transmembrane segments, as was found for pantoprazole labeling [10,15]. These contain two cysteines, cysteine 813 in the luminal vestibule and cysteine 822 in the sixth transmembrane domain.

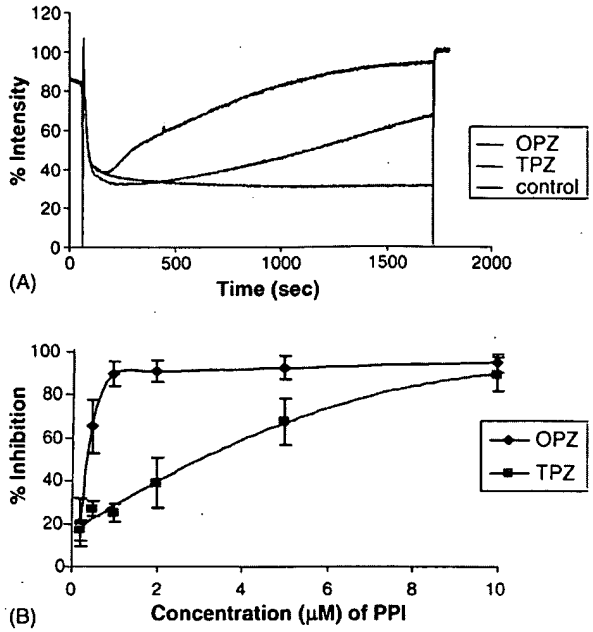


Fig. 2 – Inhibitory activity of tenatoprazole and omeprazole. (Panel A) Acridine orange uptake of the gastric H^+,K^+-ATPase under acid transporting condition as described in Section 2. Decrease of fluorescence indicates acridine orange uptake in the gastric vesicle, which is induced by inside-acidification of the sealed vesicles. The rate of inhibition by omeprazole is faster than that of tenatoprazole under these conditions. (Panel B) The inhibition by omeprazole and tenatoprazole at different concentrations. OPZ and TPZ represent omeprazole and tenatoprazole, respectively. The calculated IC_{50} for omeprazole is 0.4 μM and for tenatoprazole is 3.2 μM. The results were expressed as mean values ± S.D. of at least three experiments.

3.1.4. *Thermolysin digestion of tenatoprazole-labeled peptide fragment*

In order to identify the cysteine labeled by tenatoprazole, the labeled TM5/6 segment was cut from the gel. Tenatoprazole labeling was done with either ^{14}C-(R,S)-tenatoprazole, ^{14}C-(R)-tenatoprazole, or ^{14}C-(S)-tenatoprazole. The peptide was eluted from the gel and was further digested with thermolysin. A fragment of 3.5 kDa due to thermolysin digestion, having N-terminal sequence, NIPE, retains about half of original tenatoprazole labeling compared to the fragments of 5.6 and 6 kDa, having N-terminal sequence, SIAY and NIPE, respectively, before thermolysin digestion. The rest of the counts eluted off the gel since they were present in a very small fragment cleaved off by thermolysin. Fig. 4 shows a typical PVDF membrane pattern after transblotting from the gel and the counts of ^{14}C-tenatoprazole of each 1 mm gel slice from the top to the bottom in dotted area. All enantiomers of tenatoprazole provide very similar results (data not shown).

Table 1 – In vitro inhibition binding and inhibition by tenatoprazole

	TPZ (nmol mg^{-1} of gastric HK-ATPase)[a]	%Inhibition[a]
(R,S)-Tenatoprazole	2.56 ± 0.08	87.8 ± 1.2
(R)-Tenatoprazole	2.66 ± 0.21	88.3 ± 1.9
(S)-Tenatoprazole	2.56 ± 0.06	88.8 ± 3.3

[a] The stoichiometry and %inhibition were determined by averaging the results of three experiments.

3.2. *In vivo inhibition by tenatoprazole*

3.2.1. *Residence time of tenatoprazole on the gastric H^+,K^+-ATPase in fasting rats*

Previous in vivo data have shown that omeprazole and pantoprazole bind with maximum binding of 2.7 nmol of omeprazole or pantoprazole mg^{-1} of the H^+,K^+-ATPase at 1 h-postdose [10]. Tenatoprazole provided slow activation in vivo, which was predicted by its chemical activation rate [3]. Maximum binding of tenatoprazole was achieved at 2 h at 2.94 ± 0.47 nmol mg^{-1} of total H^+,K^+-ATPase as shown in Fig. 5. After maximum binding of tenatoprazole, tenatoprazole binding up to 24 h-postdose in vivo could be described by a first exponential equation,

$$C_{(TPZ)} = 1.824\,e^{-0.176t} + 1.141.$$

This first component is mainly from a fast decay with a half-life 3.9 h and the plateau of 1.141 at 24 h-postdose is related to binding that decays with a half-life corresponding to protein turnover (~54 h) [10,18]. Stimulated and resting membrane fractions were isolated as previously described [11,19,20] and the binding stoichiometry of each was measured. No significant difference was found (data not shown).

3.2.2. *Effect of reducing agents on tenatoprazole binding to the H^+,K^+-ATPase*

Given the likely tertiary structure of the catalytic subunit of the ATPase, cysteine 813 that is present in the luminal vestibule of the pump should be readily accessed by an in vivo reducing agent such as glutathione that is present in parietal cells at about 3.3 μmol g^{-1} of wet tissue [21]. In contrast, cysteine 822 is located 2.5 turns (~8 Å) into the α helix of transmembrane 6 in the membrane domain and is likely to be inaccessible to reducing agents.

Fig. 6 shows that GSH can differentially remove tenatoprazole or omeprazole radioactivity from the H^+,K^+-ATPase following in vivo labeling. Only 16% of omeprazole counts were not removed by GSH [9,10]. The GSH stable portion of tenatoprazole labeling was 33.6 ± 1.2%.

3.2.3. *Recovery of H^+,K^+-ATPase activity by DTT reduction of PPI-inhibited enzyme*

Tenatoprazole was intravenously administered to male rats and gastric acid secretion was shown to be fully inhibited. The gastric H^+,K^+-ATPase was isolated and enzyme activity was measured. About 20–30% of enzyme activity was observed even though acid secretion in vivo was fully inhibited. Residual enzyme activity may represent the portion of the

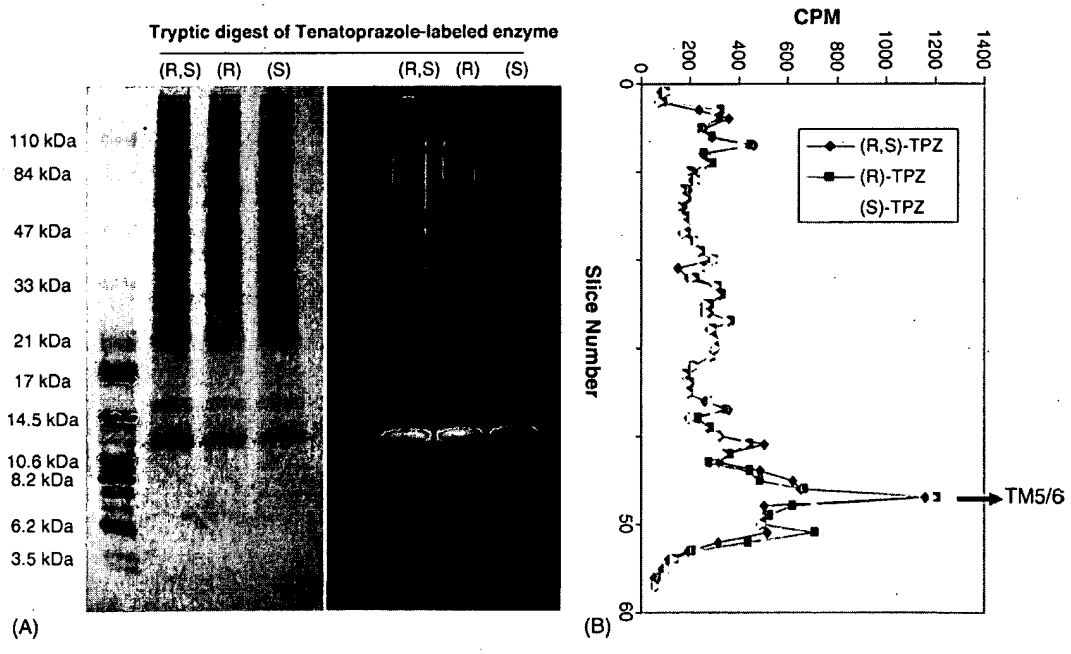


Fig. 3 – Trypsin digestion of tenatoprazole-labeled H^+,K^+-ATPase. Tenatoprazole was allowed to label the isolated gastric H^+,K^+-ATPase as described in Section 2.4.4. (Panel A) The SDS-PAGE of the Coomassie-stained trypsin digest (left) and the corresponding fluorescence of the F-MI labeled peptide fragments (right). (*R,S*)-, (*R*)-, and (*S*)-TPZ represent trypsin digest of the gastric vesicles labeled by either the racemate of tenatoprazole or (*R*)- and (*S*)-tenatoprazole, respectively. (Panel B) [^{14}C] counts per each slice of gel lane. The TM5/6 fragment is the only membrane component labeled by tenatoprazole.

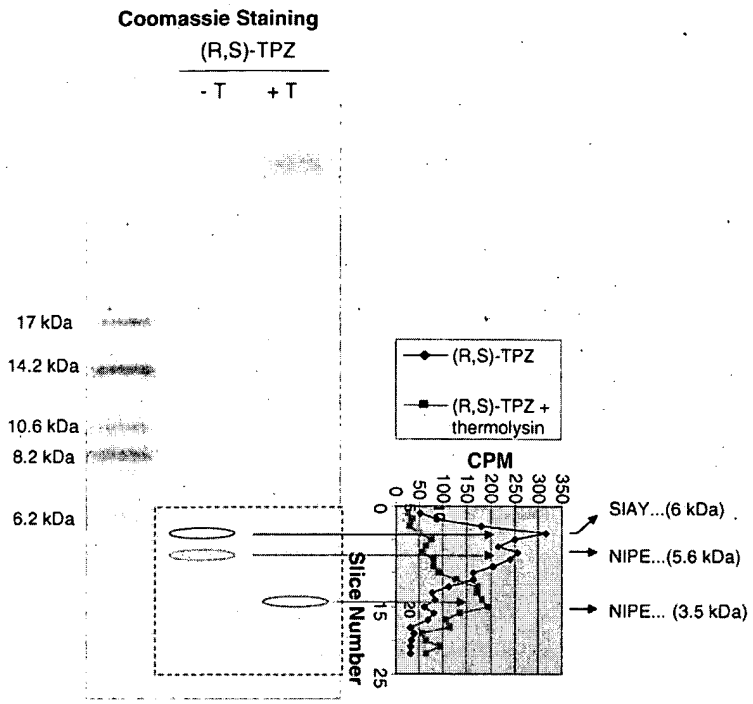


Fig. 4 – Thermolysin digestion of tenatoprazole-labeled TM5/6 fragment. −T represents the lane without thermolysin digestion and +T represents thermolysin-digested data. The dotted area was sliced and counted and sequenced. Blue circles represent the fragments before digestion and the red circle represents digested fragment.

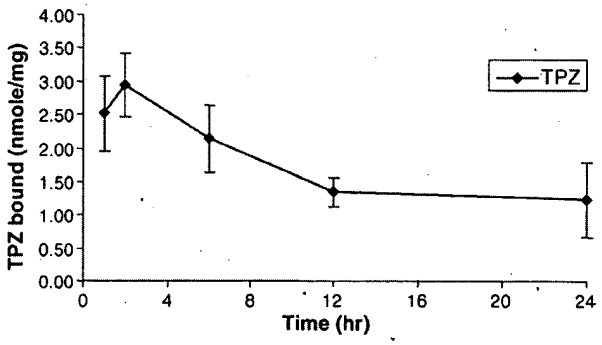


Fig. 5 – Time course of loss of tenatoprazole binding to the H^+,K^+-ATPase. At timed intervals, crude gastric membranes were collected and ^{14}C-labeling of the H^+,K^+-ATPase measured as described in experimental procedures using fasted rats. Radioactive ^{14}C-tenatoprazole was administered by IV injection at 0.1 mCi kg^{-1} of animal with a dosage of 20 μmol kg^{-1} (N = 4–6 per each group) through the tail vein. The rats were sacrificed at 1, 2, 6, 12, and 24 h. 'TPZ' represents tenatoprazole-bound gastric membrane. Diamonds (◆) represent average amounts of tenatoprazole binding per milligram of the H^+,K^+-ATPase in total membrane fraction at each given time point. The results were expressed as mean values ± S.D. Error bars represent the standard deviation of amounts of tenatoprazole binding (N = 4–6 for each time point).

enzyme located in the cytoplasmic tubules or tubulovesicles that would not be inhibited by a PPI since this compartment is not making acid.

DTT treatment of omeprazole-labeled enzyme resulted in full recovery of activity after 60 min [9] but DTT did not restore the enzyme activity inhibited by tenatoprazole. There was no difference in reversibility of the different forms of tenatoprazole, (*R*)-, (*S*)-, or (*R,S*)-tenatoprazole (Fig. 7). Glutathione also did not reverse inhibition by tenatoprazole.

3.2.4. *Trypsin digestion of in vivo tenatoprazole-labeled gastric membranes*

From trypsin digestion of C^{14}-tenatoprazole-labeled gastric membranes (Fig. 8), only TM5/6 fragment has the inhibitor bound as found for in vitro labeling.

3.3. *Pharmacokinetics of (S)-tenatoprazole sodium salt hydrate form and (S)-tenatoprazole free form*

The bioavailability of (S)-tenatoprazole free form was compared to (S)-tenatoprazole sodium salt hydrate form in vivo. This study showed (S)-tenatoprazole sodium salt hydrate provided a higher C_{max} and AUC than (S)-tenatoprazole free form. The pharmacokinetic results of the dog study are displayed in Table 2. The plasma level of tenatoprazole as a function of time is shown in Fig. 9.

The difference in bioavailability can be explained by the different solubility of the sodium salt of the S-form which in turn can be explained by the difference in the crystal structure.

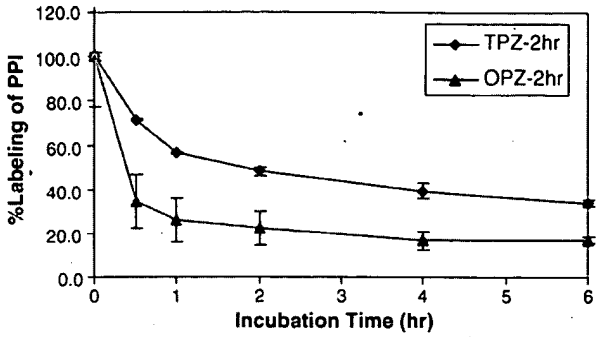


Fig. 6 – Removal of tenatoprazole or omeprazole binding by glutathione. Crude gastric membranes, prepared at 2 h after IV injection of ^{14}C-labeled drug, were incubated with 10 mM GSH and the amount of labeled H^+,K^+-ATPase was determined as described in the experimental procedure. The percent labeling at the different times of GSH incubation is shown. The points are average of percent PPI binding from five experiments at each given time point and the error bars at each time point represent the standard deviation. TPZ and OPZ represent tenatoprazole and omeprazole, respectively. OPZ-2 h or TPZ-2 h represents the enzyme preparation of 2 h-postdose of omeprazole or tenatoprazole, respectively. The results were expressed as mean values ± S.D. of five experiments.

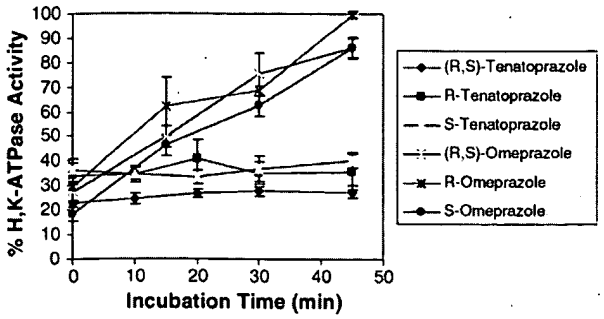


Fig. 7 – Comparison of the time dependent reversal of inhibition of tenatoprazole with omeprazole which is induced by reducing agent. Racemic (R,S)-; S-, and R-omeprazole, racemic (R,S)-, R-, and S-tenatoprazole were compared using ex vivo PPI-inhibited H^+,K^+-ATPase. An aliquot of the PPI-inhibited enzyme in vivo was incubated with 10 mM DTT (37 °C), and its activity assayed at different times after DTT addition. Activity was calculated as the percent of the activity of control ATPase which was not inhibited. The results were expressed as mean values ± S.D. of at least three experiments.

3.4. *Crystal structures of (S)-tenatoprazole free form and (S)-tenatoprazole sodium salt hydrate and their solubility*

The crystal form of (S)-tenatoprazole sodium salt hydrate was quite different from that of (R,S)-tenatoprazole sodium salt. In the crystal of (S)-tenatoprazole sodium salt hydrate, two different packing structures of molecules were observed (Fig. 10). One packing expanded in the x-axis and the other packing expanded in the y-axis of Fig. 10. This latter packing is loose, resulting in rapid water access and hence greater solubility.

(S)-Tenatoprazole was crystallized from ethyl acetate containing trace of water. Crystalline packing of molecules is shown in Fig. 11. The imidazopyridine rings overlapped each other to form a crystal exposing a hydrophobic surface.

Solubility of (S)-tenatoprazole sodium salt hydrate was 65 mg ml^{-1} of water, while the free form of both (R,S)- and (S)-tenatoprazole was almost insoluble in water due to exposure of the hydrophobic face and limited access of water.

4. Discussion

Proton pump inhibitors such as omeprazole, lansoprazole, rabeprazole, pantoprazole, and tenatoprazole are prodrugs. All proton inhibitors are converted to active sulfenamide and/or sulfenic acid by acid, which binds the gastric H^+,K^+-ATPase resulting in acid secretion inhibition. PPIs have different conversion rates depending on pH [3]. At pH 1.3, half-lives of chemical conversion to active form(s) in the absence of thiol agents were 4.7 min for omeprazole, 3.2 min for lansoprazole, 9.3 min for pantoprazole, and 12.8 min for tenatoprazole. At pH 2.3, half-lives of PPIs were observed as 7.3 min for omeprazole, 5.3 min for lansoprazole, 13 min for pantoprazole, and 25.9 min for tenatoprazole [3]. This suggests that, under acid secretion condition, tenatoprazole is slower in converting into the active form in stimulated parietal cell.

4.1. *In vitro study of the inhibition of tenatoprazole in comparison with omeprazole*

Inside-out sealed gastric vesicles prepared from hog stomach can create a pH gradient of about 4 pH units across the pump membrane [22,23]. Acidification of the vesicle inside to about pH 3 by K^+/H^+ exchange across gastric membranes enables PPI activation and labeling, resulted in inhibition of the pump activity in the acid-transporting gastric vesicles by binding to luminally accessible cysteines [13,24,25]. Measuring acridine uptake of the gastric vesicles in the presence of PPIs provides an estimate of the rates of PPI activation, even though acridine orange uptake reduces the activation rate of PPI due to interior buffering by acridine orange [25]. When acridine orange uptake under acid transporting condition is measured as a

Table 2 – Pharmacokinetic parameters of (S)-tenatoprazole free form and sodium salt hydrate form

Dose	T_{max} (h)	C_{max} (ng ml^{-1})	$AUC_{t(0-8\ h)}$ (ng h ml^{-1})
(S)-Tenatoprazole Na salt (100 mg kg^{-1})	1.3	183 021	822 785
(S)-Tenatoprazole free form (100 mg kg^{-1})	2.5	104 751	434 017

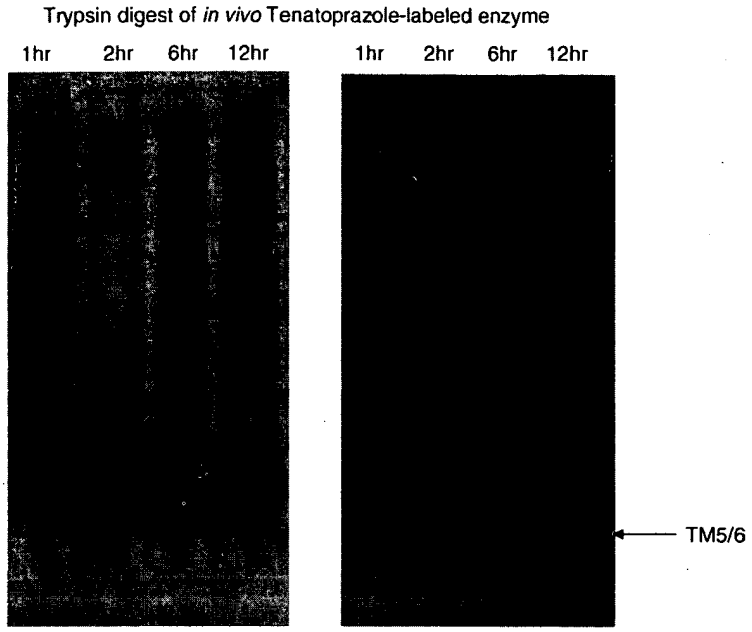


Fig. 8 – Autoradiogram of trypsin digest of the gastric membrane prepared at timed intervals after PPI treatment in vivo. After isolation of the labeled membranes, they were digested by trypsin as described in Section 2.5.5. and the digest separated by SDS-PAGE to identify the labeled region of the ATPase. Left panel is a typical Coomassie-stained PVDF membrane and right panel is a typical autoradiogram of trypsin digest. Each lane indicates digestion of tenatoprazole-labeled sample prepared for different lengths of time. TM5/6 represents a peptide fragment containing the fifth and sixth transmembrane segments. Tenatoprazole was labeled only at TM5/6 in vivo.

function of time, this showed that inhibition of tenatoprazole was slower than that of omeprazole (Fig. 2, Panel A). The IC_{50} of tenatoprazole and omeprazole at medium pH 6.6 was 3.2 and 0.4 μM, respectively (Fig. 2, Panel B). The gastric H^+,K^+-ATPase can generate a pH of about 3.0 inside the gastric membrane vesicles. At pH 3.1, half-lives of omeprazole and tenatoprazole were 9 and 30 min, respectively [3]. This slow activation of tenatoprazole compared to that of omeprazole explains why tenatoprazole restored the fluorescence of acridine orange slowly and why the IC_{50} for tenatoprazole is higher than that of omeprazole in isolated gastric H^+,K^+-ATPase.

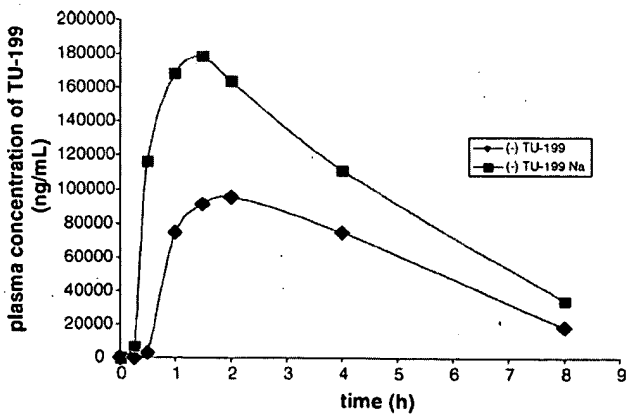


Fig. 9 – The plasma level of tenatoprazole after oral administration. In dogs (N = 3), a dose (100 mg kg^{-1}) of (S)-tenatoprazole free form or sodium salt was administrated orally using a capsule contained the compounds as crystallized powder. (–) TU-199 and (–) TU-199 Na represent (S)-tenatoprazole free form and (S)-tenatoprazole sodium salt hydrate form. The results were expressed as mean values.

4.2. *Identification of the cysteine sites of the proton pump, where tenatoprazole is covalently bound*

Tenatoprazole binds at the catalytic subunit of the gastric acid pump with a stoichiometry of 2.6 nmol mg^{-1} of the enzyme in vitro. In vivo, maximum binding of tenatoprazole was 2.9 nmol mg^{-1} of the enzyme. Binding stoichiometry of tenatoprazole in vitro and in vivo was very similar to those of rabeprazole, omeprazole, and pantoprazole [10,17].

The catalytic subunit of the gastric H^+,K^+-ATPase has 10 transmembrane segments and several cysteines accessible from the exoplasmic surface, the site of activation of the PPIs. While all PPIs reacted with cysteine 813 at the exoplasmic vestibule entry into sixth transmembrane segment (TM6) of the alpha subunit of the pump, omeprazole was also able to bind to cysteine 892 in the loop between seventh transmembrane segment (TM7) and eighth transmembrane segment (TM8), lansoprazole with cysteine 321 at the end of third transmembrane segment (TM3) and pantoprazole reacted also with cysteine 822 deeper within the membrane domain of TM6 [10,13,15,16,26]. The binding domain of tenatoprazole was TM5/6, which contains Cys813 and Cys822. Thermolysin digestion of TM5/6 labeled by tenatoprazole shows that

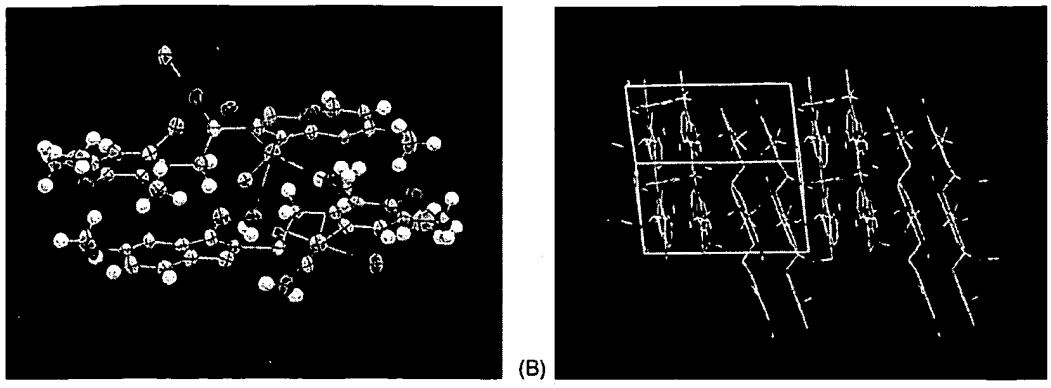


Fig. 10 – Crystalline molecular packing of (*S*)-tenatoprazole sodium salt hydrate. (Panel A) Crystal structure of (*S*)-tenatoprazole sodium salt hydrate and (Panel B) unit cell of the crystals.

tenatoprazole labeled both Cys813 and Cys822, as previously found for pantoprazole labeling [10].

4.3. *In vivo labeling of tenatoprazole on the gastric H^+,K^+-ATPase*

No reversal of the inhibited pump by PPIs is seen even after overnight incubation of the inhibited enzyme in vitro [27–29]; hence, the disulfide bonds are stable in the absence of disulfide reducing agents. If synthesis of new pump protein is the only means of generation of active enzyme, in vivo, covalent inhibition of the enzyme would be reversed only by de novo pump synthesis. In vivo, the half-life for synthesis of the catalytic subunit under control conditions or with omeprazole inhibition has been shown to be ~54 h [18]. However, half-lives of recovery of acid secretion with decay of PPI binding were much shorter than that of de novo pump synthesis [18].

The decay of omeprazole and pantoprazole binding was composed of two components. One was a fast decay and the other was a slow decay with kinetics similar to the pump enzyme turnover. Most omeprazole binding (about 84%) and half of pantoprazole binding decayed with a half-life 6 h [10].

Maximum binding of tenatoprazole in vivo was obtained at 2 h after IV administration, while omeprazole and pantoprazole maximized at 1 h [10]. Stoichiometries of tenatoprazole binding were 2.52 ± 0.56 nmol mg^{-1} of the enzyme at 1 h and 2.94 ± 0.47 nmol mg^{-1} of the enzyme at 2 h. Stoichiometry at 1 h was lower than that of sample at 2 h, which suggests that tenatoprazole is slowly activated and bound to the ATPase. The final stoichiometry of maximum binding of tenatoprazole in vivo was very similar to those of omeprazole and pantoprazole. Decay of tenatoprazole binding on the gastric H^+,K^+-ATPase was composed of two components. One was fast decay with a half-life 3.9 h and the other was a plateau, which must be derived by slow decay similar to the protein turnover as shown in pantoprazole decay. Omeprazole is known to bind initially in the canaliculus at the site of acid secretion [30,31]. This suggests that tenatoprazole also binds the pump enzyme at the canaliculus. However, redistribution of the ATPase [32] after tenatoprazole binding enables arrival of tenatoprazole-bound enzyme into the microsomal membrane fraction.

4.4. *Reversibility of the binding of tenatoprazole to the ATPase*

The time course of tenatoprazole labeling shows maximum binding at 2 h (Fig. 5). The sample prepared at 2 h showed 66% cleavage of tenatoprazole labeling by GSH, which is 1.95 nmol mg^{-1} of the enzyme. Irreversible labeling (34% of total labeling) was 0.99 nmol mg^{-1} of the enzyme (Fig. 6). It is

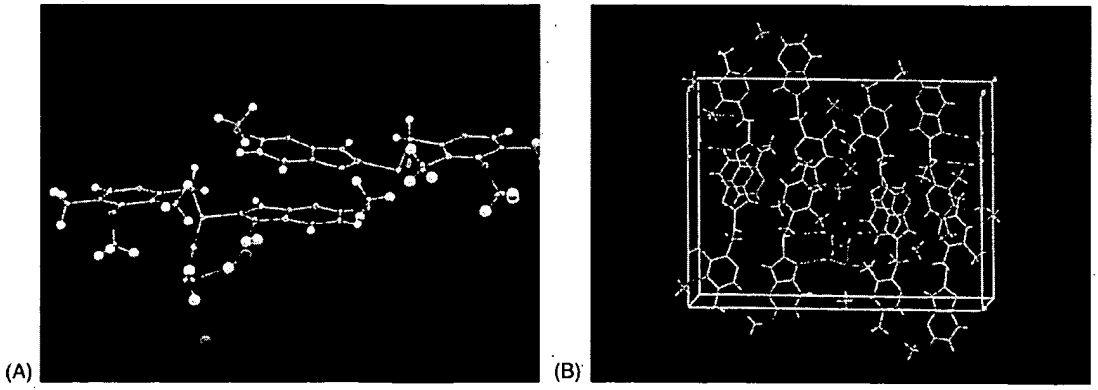


Fig. 11 – Crystalline packing of (*S*)-tenatoprazole free form with unit cell. (Panel A) Crystal structure of (*S*)-tenatoprazole free form and (Panel B) unit cell of the crystal.

known that GSH-sensitive labeling site is Cys813 and that the GSH-insensitive site is Cys822 [9,10]. This result suggests that 66% of tenatoprazole binds at Cys813 and 34% of tenatoprazole binds at Cys822.

The enzyme at 2 h had about 20–30% enzyme activity (Fig. 7), which suggests about 20–30% of the enzyme was not labeled under this experiment condition. Non-labeling of PPI suggests that about 20–30% of the enzyme is located in the resting tubulovesicles or tubules when PPI was administrated. However, a reducing agent did not recover the activity of tenatoprazole-labeled enzyme, while it enabled to recover the activity of omeprazole-labeled enzyme (Fig. 7). Difference among the racemates, (R)-, and (S)-PPIs does not provide any differences of activity recovery. The stereo-specificity of PPI does not affect acid-derived chemical activation of PPI; therefore, it does not affect binding sites. PPI-binding at Cys822 is not reversed by GSH or DTT reduction and inhibitory activity is retained [9,10]. Non-recovery of tenatoprazole-inhibited enzyme and GSH-insensitive tenatoprazole binding site also shows that tenatoprazole binds at Cys822 together with at Cys813. Both cysteines are in the transport pathway of the enzyme and TM6 conformational changes are required for enzyme cycling; hence, binding at either cysteine 813 or 822 is sufficient for full enzyme inhibition [33].

4.5. *In vivo labeling site of tenatoprazole*

Tenatoprazole labeling in vivo was only at TM5/6 as was found in vitro. Omeprazole in vivo labeling occurred mostly at TM5/6, then some was found at TM7/8 at cysteine 892 which is distant from the transport domain of the ATPase [10]. In vivo labeling of tenatoprazole was very similar to that of pantoprazole being seen only at TM5/6 [10,15]. Tenatoprazole was found to have long-lasting and potent effects on gastric acid secretion [4]. This long-lasting activity must be due to either non-reversibility by glutathione like pantoprazole binding or long-lasting plasma half-life. In this study it is shown that one aspect of the long-lasting activity of tenatoprazole is due to binding of tenatoprazole at Cys822.

4.6. *Pharmacokinetics of (S)-tenatoprazole free form and sodium salt hydrate form related with crystal structure and solubility*

(S)-Tenatoprazole sodium salt hydrate form provided almost twice the AUC as compared to the free form in dogs. In this experiment, the dog studies were performed with a similar capsule formulation; the capsules contained the compounds as crystallized powder without excipients. The difference in AUC could be explained by the fact that the crystal structure and hydrophobic nature of the powder of free acid may have resulted in clusters of material to be formed, which would delay the access of water molecules to the drug. The free from of tenatoprazole is almost insoluble in water. Poor solubility of free form in water may reduce homogenous contact to the intestine cell layer, resulting in poor absorption compared to sodium salt. The crystal structure of (S)-tenatoprazole free form shows hydrophobic imidazopyridine and pyridine are exposed outside, resulting in very poor solubility in water due to limited water access. The crystal structure of (S)-tenatoprazole sodium salt hydrate shows good exposure of the sodium atoms to water molecules outside with a larger volume of 1841.7 $Å^3$. This larger exposure of the crystal interior to water enables better contact with the aqueous solution, increasing the solubility. The pharmacokinetics of the sodium salt compared to the free form suggest that better solubility results in better absorption, resulting in a higher AUC.

The clinical implication of these data suggests that the inhibition by tenatoprazole may be longer lasting than that of omeprazole, which might imply better night-time control of acidity by the former. However, this has not been evaluated in any properly controlled clinical studies to date.

Knowledge of the mechanism and in vitro and in vivo binding of tenatoprazole as an acid-activated prodrug allows prediction of many of its properties. Firstly, since this prodrug is a covalent inhibitor, its duration of action outlasts its presence in the blood. Since PPIs require acid activation, they are most effective when the parietal cells are stimulated, hence are usually given 30–60 min after meals. Since most of PPIs such as omeprazole, lansoprazole, and rabeprazole generally have a short plasma half-life (60–90 min) [34] and not all acid pumps are active at any one time, their effect is cumulative, reaching steady state on once a day dosing by the third day, inhibiting about 70% of all pumps. However, the half-life of tenatoprazole is about 9.3 h in human, which enables to reach steady state in shorter time compared to other PPIs [6–8]. If there is significant acid secretion at night resulting in night-time GERD, it is difficult to control this with currently available PPIs. The long half-life of tenatoprazole in the plasma combined with slow decay of tenatoprazole binding to the pump may enable prolonged duration of acid suppression to control nocturnal acid breakthrough.

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